RXRA induces endoplasmic reticulum stress via ATP2A3 transcriptional modulation in ovarian cancer cells

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Submitted: 19 August 2024; **Accepted:** 1 January 2025
Online publication: 4 March 2025

Arch Med Sci
DOI: https://doi.org/10.5114/aoms/199701

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Abstract

Introduction: Ovarian cancer (OV) progression involves intricate interactions of genes and cellular pathways, with the ATP2A gene family recently emerging as significant.

Material and methods: Through bioinformatic analysis, we evaluated the expression and prognostic impact of the ATP2A gene family in various cancers, emphasizing OV. Immunohistochemical and clinical expression profiles of ATP2A2 were subsequently analyzed in OVs. We further explored the effects of ATP2A3 modulation on cellular behaviors, involving proliferation, apoptosis, migration, and invasion in OV cell lines. The function of ATP2A3 in mediating endoplasmic reticulum (ER) stress and its influence on calcium-mediated kinase activities was elucidated. Furthermore, a comprehensive analysis of The Cancer Genome Atlas (TCGA) was conducted, spotlighting the interplay between RXRA and ATP2A3. Finally, their effects on OV cell progression were analyzed *in vitro*.

Results: Our results highlighted a consistent association between low expression of ATP2A3 in OV and improved patient prognosis. ATP2A3 regulation has significant effects on the proliferation, apoptosis, migration, and invasion of OV cells. Notably, overexpression of ATP2A3 enhanced ER stress biomarkers but inhibited calcium-mediated kinase activity. At the same time, *in vitro* cell experiments found that RXRA overexpression can inhibit the malignant behavior of OV cells, and it is positively correlated with ATP2A3. Overexpression of RXRA inhibited OV progression by inducing the ER stress response, which was partially offset by ATP2A3.

Conclusions: ATP2A3 and RXRA synergistically modulate OV cell behaviors and the ER stress response, revealing prospective therapeutic avenues for OV intervention.

Key words: ovarian cancer, ATP2A3, RXRA, endoplasmic reticulum stress, cellular behaviors.

Introduction

Ovarian cancer (OV) is the seventh most frequent malignancy among women worldwide and the eighth major cause of cancer-related death,

posing a significant threat to the health of women [1, 2]. While incidence rates have been observed to decrease steadily in high-income countries, there is an alarming rise in many low- and middle-income countries [3]. OV can be categorized based on its characteristics and origin into ovarian epithelial cancer, malignant sex cord-stromal tumors, ovarian malignant germ cell tumors, and metastatic tumors [4, 5]. Alarmingly, 70% of OV cases are detected at a late stage, making treatment exceedingly challenging. Presently, while conventional surgery and chemotherapy offer temporary relief from OV [6], recurrence typically occurs within 2–3 years of treatment. The frequent need for repeated surgery and chemotherapy not only hampers the therapeutic progress but also severely diminishes the quality of life for patients [7]. Given this, identifying reliable biomarkers for the clinical diagnosis and treatment of ovarian cancer is of paramount importance.

Endoplasmic reticulum (ER) stress is a critical cellular response implicated in various pathophysiological conditions, including cancer [8]. Perturbations in ER homeostasis, from a variety of causes such as nutrient deprivation, oxidative stress, and imbalanced calcium levels, can lead to ER stress and subsequently activate the unfolded protein response (UPR) [9, 10]. Central to the modulation of ER homeostasis is the calcium signaling pathway, specifically governed by the ATP2A gene family [11]. These genes are crucial for maintaining low cytoplasmic concentrations of Ca^{2+}, a key factor not only in tumorigenesis but also in inducing ER stress [12]. For instance, the ATP2A1 gene encodes an enzyme vital for reabsorbing cytosolic Ca^{2+} into the sarcoplasmic reticulum, thus influencing striated muscle functions [13]. Similarly, ATP2A2 plays a quintessential role in maintaining reduced cytoplasmic levels of Ca^{2+} ions [14], while the ATP2A3 gene encodes a protein that functions as an intracellular pump predominantly found within the sarcoplasmic or endoplasmic reticulum (ER) of muscle cells [15, 16]. Recent oncological research has shed light on the relevance of the ATP2A gene family in cancer biology. For instance, the potential effects of resveratrol on breast cancer progression are thought to be mediated through modulating ATP2A3 expression [17]. Additionally, germline mutations in ATP2A2 are associated with lung and colon cancers, suggesting an underlying dysfunction in ATP2A2 [18]. Collectively, these findings underscore the integral role of the ATP2A gene family in calcium regulation as well as their potential implications in the pathogenesis and progression of various cancers.

Although the importance of calcium signaling and the ATP2A gene family in tumorigenesis is well recognized, the role of the transcription factor RXRA in OV remains understudied. Preliminary data suggest a possible link between RXRA and ATP2A3, pointing to potential regulatory mechanisms that may influence the pathogenesis of OV. However, the precise interaction, as well as the cellular consequences and larger ramifications, remain unknown.

This study aims to uncover new insights into the interaction between RXRA and ATP2A3 in OV, which may be critical to advancing diagnostic and treatment strategies for this tumor.

Material and methods

Analysis of ATP2A family gene expression and prognostic in OV using multigroup databases

We utilized the UALCAN database (http://ualcan.path.uab.edu/index.html) to analyze the expression levels of ATP2A family genes (ATP2A1, ATP2A2, ATP2A3) across 33 pan-cancer types based on TCGA transcriptome data. Specifically, for ovarian serous cystadenocarcinoma (OSC), we used the TCGA-0V dataset (dbGaP Study Accession: phs000178.v11. p8) containing 376 tumor samples. Normal control samples (n = 180) were obtained from the GTEx dataset (https://gtexportal.org/) using version V8. All data were analyzed via the Gene Expression Profilin Interactive Analysis (GEPIA) server. To assess the statistical significance of differences in expression levels, we employed the non-parametric Wilcoxon rank sum test. To ascertain the prognostic implications of the ATP2A gene family in OV, Kaplan-Meier (KM) survival curves were generated, segmenting based on high and low gene expression for post-progression survival (PPS), overall survival (OS), and progression-free survival (PFS). The log-rank test was used to assess differences in survival curves, with a significance level of $p < 0.05$ specified.

Immunohistochemical and proteomic assessment of ATP2A3

To elucidate the expression patterns of ATP2A3 in OV, an integrative approach encompassing immunohistochemical and proteomic methodologies was adopted. Initially, immunohistochemical staining data for ATP2A3 in both normal and tumor ovarian tissues were retrieved from the Human Protein Atlas (HPA) database (https://www.Proteinatlas.org/). The specific dataset CAB010882 was utilized to ensure a thorough examination of ATP2A3 protein expression patterns. Subsequently, leveraging the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database (https://cptac-data-portal.georgetown.edu/), we extracted proteomic data pertinent to ATP2A3 expression across various clinical subgroups of OV, delineated by stage, race, age, and grade. This data compilation facilitated a comprehensive assessment of

ATP2A3 protein expression modulation as a function of diverse ovarian cancer clinical attributes.

Comprehensive analysis of prognostic genes associated with ATP2A3 in OV

Transcriptome data were collected from TCGA and GTEx databases, including 376 OSC and 180 normal samples, and differentially expressed genes (DEGs) were subsequently identified using the limma package in R. Criteria were a fold change (FC) greater than 2 or less than 0.5 with a *p*-value threshold < 0.05. Subsequently, we downloaded a dataset of 707 prognosis-related genes associated with OSC from the TCGA database. Concurrently, the High-Throughput Functional Target (HTFtarget) database (http://bioinfo.life.hust.edu.cn/hTFtarget) furnished a list of 157 target genes linked to ATP2A3. Employing the VennDiagram package in R, we systematically identified overlapping genes from the cohort of 707 prognostic genes, the 157 target genes, and the downregulated DEGs from TCGA. From this intersection, we pinpointed a hub gene. To attain a nuanced understanding, the expression profile of the identified hub gene in OV was investigated using the GEPIA server. Moreover, its potential prognostic significance in OV was assessed by constructing KM survival curves and analyzing its association with PFS among OV patients. Finally, a Spearman correlation analysis was performed to evaluate the relationship between expression of the hub gene and ATP2A3, offering insights into their synergistic role in the context of OV.

Cell culture

Ovarian cancer is a heterogeneous disease with diverse molecular and genetic characteristics. Using multiple cell lines allows us to account for this heterogeneity and to ensure that our findings are not confined to a single, potentially atypical, model. We sourced four OV cell lines (A2780, OVCAR3, OVCA433, and SKOV3), alongside a normal ovarian epithelial cell line (HOSE), and the HEK 293T cell line from the American Type Culture Collection (ATCC). All these cell lines were cultured in RPMI 1640 medium enriched with 10% fetal bovine serum (FBS, Gibco) and fortified with a 1% solution of penicillin-streptomycin (Solarbio, China). They were subsequently incubated at a temperature of 37°C within a controlled environment of 5% CO_2.

Transfection and treatment assay

To modulate gene expression, we employed specific small interfering RNAs (siRNAs) designated as si-ATP2A3 #1 and si-ATP2A3 #2 to target and downregulate ATP2A3 expression in the OV cell lines. In parallel, we introduced overexpression plasmids carrying genes of ATP2A3 and RXRA into these cells to amplify their endogenous expression. Cells that underwent transfection with an empty vector were used as baseline controls. The Lipo2000 reagent (Polyplus Transfection, France) was the agent of choice for all transfection procedures, and the process adhered strictly to the manufacturer's instructions. After transfection, cells designated for specialized assays were exposed to 4-PBA at a dose concentration of 1 mM. Concurrently, to assess calcium modulation's potential impact, a subset of cells was treated with the calcium chelator BAPTA-AM, at a concentration of 10 µM.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and reverse-transcribed using a cDNA synthesis kit (DBI, Germany). The qRT-PCR reactions were executed with the SYBR Green Master kit (DBI, Germany) on an AB Fast 7500 real-time PCR system, strictly adhering to the manufacturer's protocols. The relative expression of mRNA was assessed by the $2^{-\Delta\Delta Ct}$ technique using GAPDH as an internal reference. The utilized primer sequences were: ATP2A3 forward: 5′- GCCTCTCATTTTCCAGGTGAC-3′, reverse: 5′- CTCATTTCTTCGTGCATGTGG-3′; RXRA forward: 5′-TTCGCTAAGCTCTTGCTC-3′, reverse: 5′-ATAAGGAAGGTGTCAATGGG-3′; GAPDH forward: 5′-TGTTCGTCATGGGTGTGAAC-3′, reverse: 5′-TATTTGGCAGGTTTTTCTAG-3′.

Western blotting (WB) analysis

Total protein was extracted from transfected cells using RIPA lysis buffer (Beyotime Biotechnology). SDS-PAGE was used to separate identical quantities of proteins, which were then transferred to PVDF membranes (Millipore, Merck). The membrane was mixed with primary antibodies (ATP2A3, BIP, Caspase 12, CHOP, ATF4, p-PERK, PERK, eIF2a, p-eIF2a, p-CaMKIIa, CaMKIIa, CHOP, RXRA) (1 : 1000, Abcam, USA), and GAPDH (1 : 5000, Abcam, USA) was probed overnight at 4°C. After being washed, the membrane was incubated for 1 hour at room temperature with an HRP-conjugated secondary antibody (1 : 5000, Abcam, USA). The ECL detection system (Thermo Fisher Scientific) was used to observe protein bands.

Flow cytometry assay

We used the Annexin V/FITC Apoptosis Detection Kit from BD Biosciences to assess cellular apoptosis. According to the manufacturer's guidelines, cells were collected, cleaned with cold PBS, and then stained with Annexin V-FITC and propidium iodide (PI). After that, the FACSCalibur flow

cytometer (BD Biosciences) was used to analyze the labeled cells. Data were collected and processed using the associated software, and cells in the early and late stages of apoptosis were distinguished based on the fluorescence intensity and quadrant distribution.

Assay for cell proliferation

We tested cell proliferation using the Cell Counting Kit 8 (CCK-8) kit (Qihai Biotechnology Company, China). Briefly, we incubated transfected cells in CCK-8 solution at 37°C for 2 to 4 h and cultured them in 96-well plates. After 24 h, 48 h, 72 h, 96 h, and 120 h of incubation, the absorbance (OD) of the cells in each well at 450 nm was measured with a microplate reader.

Assays for migration and invasion

Transwell chambers, both with and without Matrigel coating (8 µm pore size, Corning, USA), were employed to evaluate cell invasion and migration abilities. The transfected cells were inoculated into the upper chamber containing 200 µl of RPMI 1640 medium. The lower chamber has 20% FBS and 600L RPMI 1640 medium. We fixed migrating and invading cells with methanol and stained them with DAPI after incubation at 37°C for 48 h. Finally, we photographed and counted migrating and invasive cells under a microscope.

Dual-luciferase reporter gene assay

The Dual-Luciferase Reporter Gene Assay was performed to investigate the effect of RXRA overexpression on ATP2A3 transcriptional activity. The luciferase reporter plasmids, containing the cloned target sequences of ATP2A3 promoter regions, and the RXRA overexpression plasmid or empty vector were co-transfected into HEK 293T cells using the Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) [19–21]. The luciferase reporter plasmids contained Firefly luciferase as the primary reporter and Renilla luciferase as the control reporter for normalization. The total amount of DNA was kept constant in all co-transfections. At 48 h after transfection, cells were collected, and luciferase activities were assessed with the Dual-Luciferase Reporter Assay Kit (Promega Corporation) as per the provided guidelines. Luminescence was recorded using a luminometer.

Chromatin immunoprecipitation (ChIP) assay

To validate the direct binding of RXRA to the promoter region of ATP2A3, a ChIP assay was employed. OV cell lines were cross-linked with formaldehyde to preserve protein-DNA interactions, and then subjected to sonication to break the DNA into segments roughly between 200 to 500 base pairs long. The fragmented chromatin was subsequently immunoprecipitated with an RXRA-specific antibody. As a control, an IgG antibody was used for non-specific binding. After reversing the cross-links, the DNA was purified and subjected to qRT-PCR using primers specifically designed to amplify regions of interest within the ATP2A3 promoter. The amplification data confirmed the direct association between RXRA and the ATP2A3 promoter region, indicating the potential regulatory role of RXRA in ATP2A3 transcriptional activity.

Statistical analysis

Statistical significance for *in vitro* cell experiments was assessed using appropriate statistical tests, and all experiments were performed in triplicate or as indicated. Data are presented as means ± standard deviation (SD). When $p < 0.05$, the difference was considered to be statistically significant, and GraphPad Prism was used for statistical analysis.

Results

Expression and prognostic impact of the ATP2A gene family in OV samples

Expression analyses of ATP2A1, ATP2A2, and ATP2A3 were conducted in 33 tumor specimens. Notably, ATP2A1 demonstrated reduced expression in the majority of tumor samples, while ATP2A2 exhibited pronounced expression. In contrast, ATP2A3 expression varied considerably across different tumor samples (Supplementary Figures S1 A, C, Figure 1 A). Further database analysis confirmed that ATP2A1, ATP2A2, and ATP2A3 were all expressed at low levels in OV tissues (refer to Supplementary Figures S1 B, D, and Figure 1 B). KM survival analysis was utilized to decipher the impact of the ATP2A gene family on the prognosis of OV patients. In OV samples with elevated ATP2A1 expression, the probability of PFS decreased, the probability of OS increased, and the probability of PPS slightly decreased (Supplementary Figures S2 A–C). Conversely, high ATP2A2 expression in OV samples was associated with poorer PFS probability but higher OS and PPS probability (Supplementary Figures S2 D–F). Furthermore, as shown in Figures 1 C–E, elevated ATP2A3 expression was consistently associated with superior PFS, OS, and PPS probabilities in OV.

Correlation between ATP2A3 protein expression and clinical features in OV patients

We subsequently investigated ATP2A3 protein expression in OV patients utilizing the HPA data-

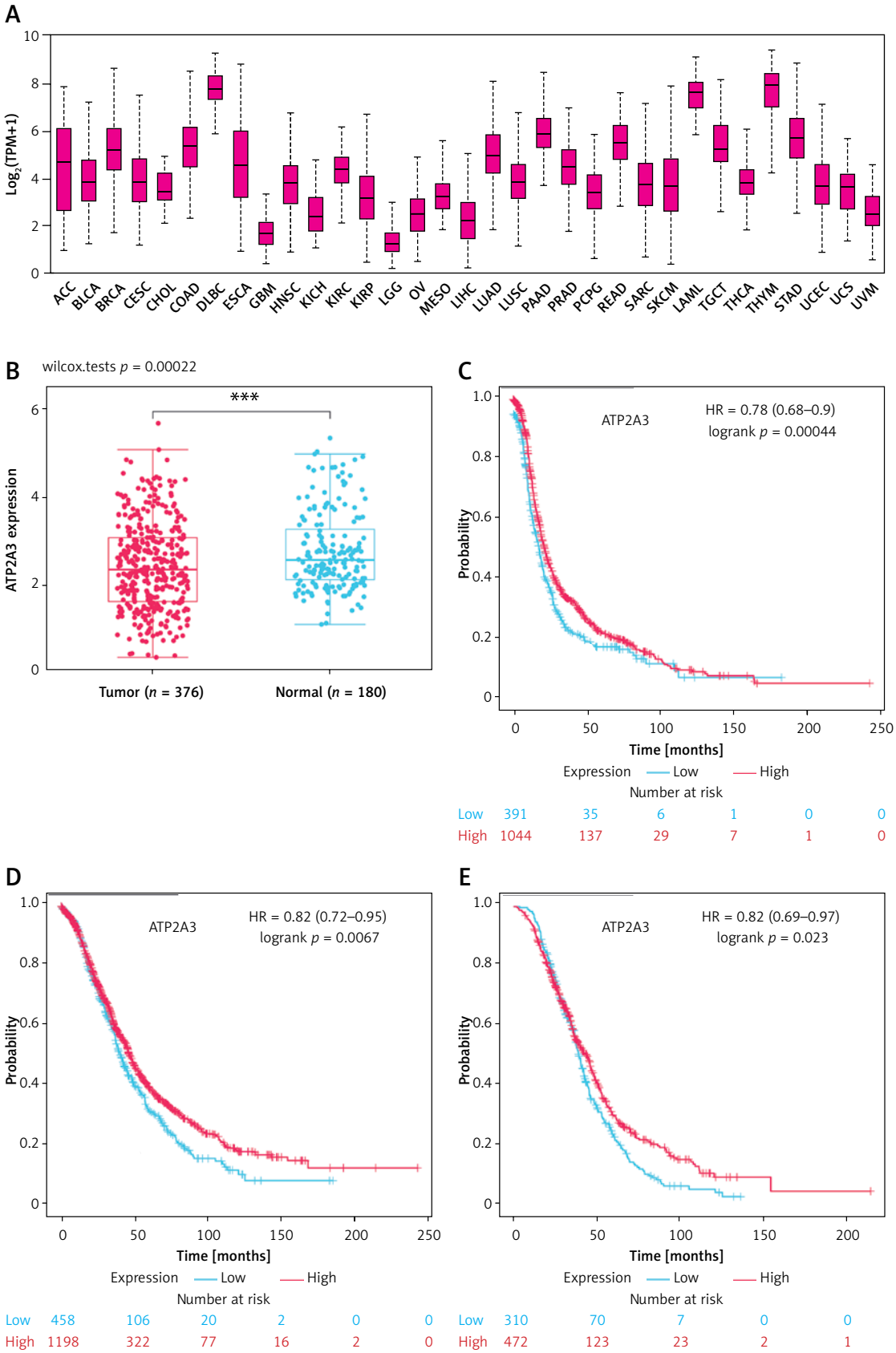


Figure 1. Expression and prognostic analysis of ATP2A3 in OV. **A** – Expression of ATP2A3 in 33 tumor samples in the UALCAN database; different box lines represent different tumor types. **B** – Wilcoxon detection of the expression pattern of ATP2A3 between TCGA-OSC samples and GTEx-control samples. ***$p < 0.001$. **C**–**E** – KM survival curve, the effect of differential expression of ATP2A3 on the probability of PFS (**C**), OS (**D**) and PPS (**E**) of patients

base. As depicted in Supplementary Figure S3 A, ATP2A3 protein levels in primary tumor samples were markedly reduced compared to their normal counterparts. Further exploration, as presented in Supplementary Figures S3 B–E, revealed significant associations between ATP2A3 protein expression and various clinicopathological parameters. In terms of cancer staging, ATP2A3 protein expression showed no significant differences between stages 1 and 3. However, significant differences were apparent between stages 1 and 4, and stages 3 and 4. From the perspective of race, there was no significant difference in ATP2A3 expression between Caucasian, African-American, and Asian. Analyzing age-based groups, the variation in ATP2A3 protein levels across different age brackets was highly significant. Lastly, regarding tumor grading, a pronounced difference in ATP2A3 protein levels was observed between grades 2 and 3.

Expression of ATP2A3 and its effect on the phenotype of OV cells

qRT-PCR analysis revealed that ATP2A3 was significantly downregulated in A2780, OVCAR3, and SKOV3 cells, with particularly pronounced reductions observed in OVCAR-3 and A2780 cells (Figure 2 A), suggesting its potential tumor-suppressive role in ovarian cancer. Our data indicated that ATP2A3 expression in A2780 and OVCAR3 was notably lower compared to HOSE, which presents a more pronounced difference and thus a more suitable model for studying the effects of ATP2A3 modulation. Following this, we induced ATP2A3 overexpression and knockdown in OVCAR-3 and A2780 cells. The efficiency of these modulations was then validated through qRT-PCR and WB. Among the siRNAs used, si-ATP2A3 #2 demonstrated the most pronounced knockdown efficiency (Figures 2 B–E). We subsequently as-

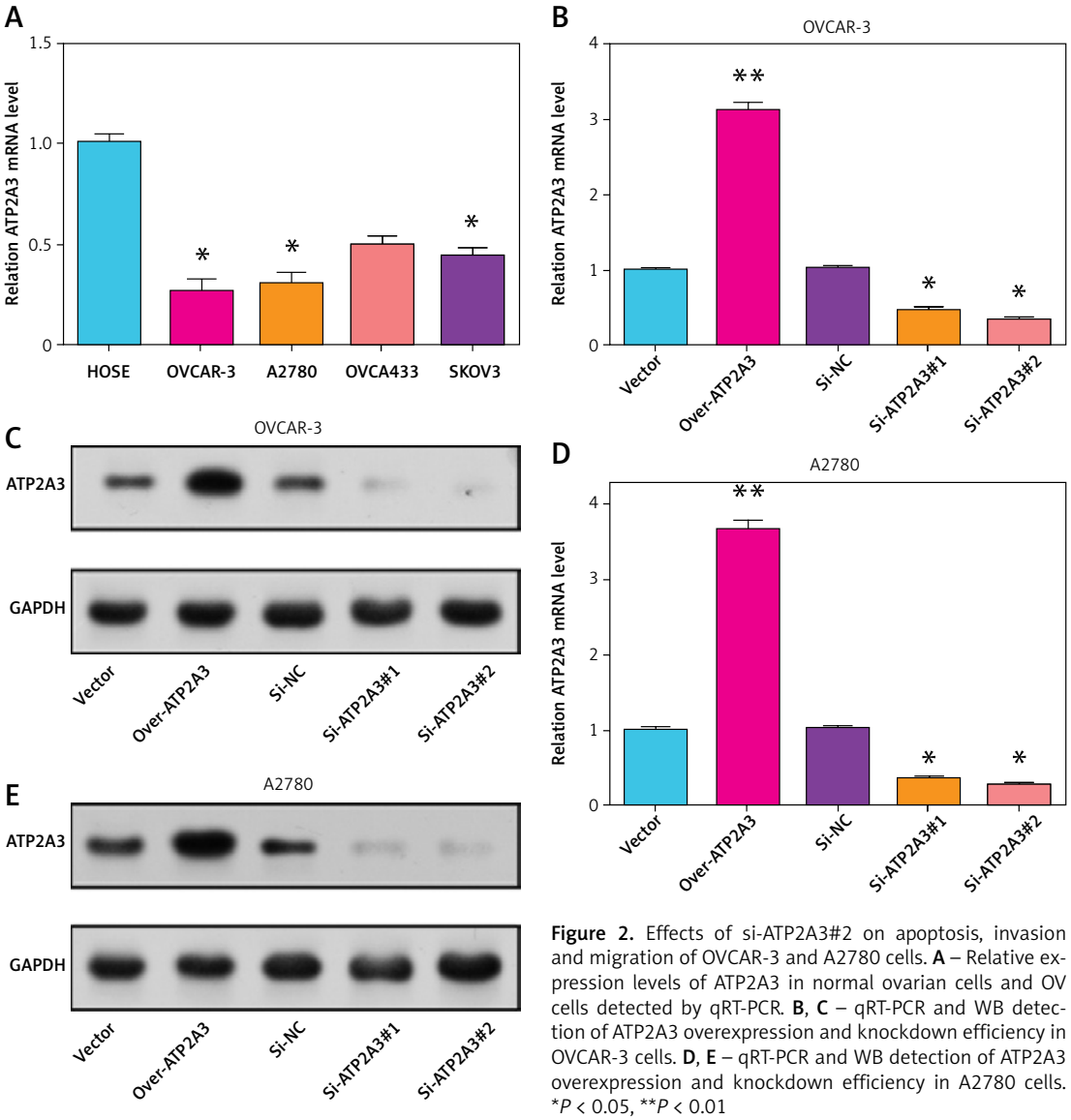


Figure 2. Effects of si-ATP2A3#2 on apoptosis, invasion and migration of OVCAR-3 and A2780 cells. **A** – Relative expression levels of ATP2A3 in normal ovarian cells and OV cells detected by qRT-PCR. **B**, **C** – qRT-PCR and WB detection of ATP2A3 overexpression and knockdown efficiency in OVCAR-3 cells. **D**, **E** – qRT-PCR and WB detection of ATP2A3 overexpression and knockdown efficiency in A2780 cells. **P* < 0.05, ***P* < 0.01

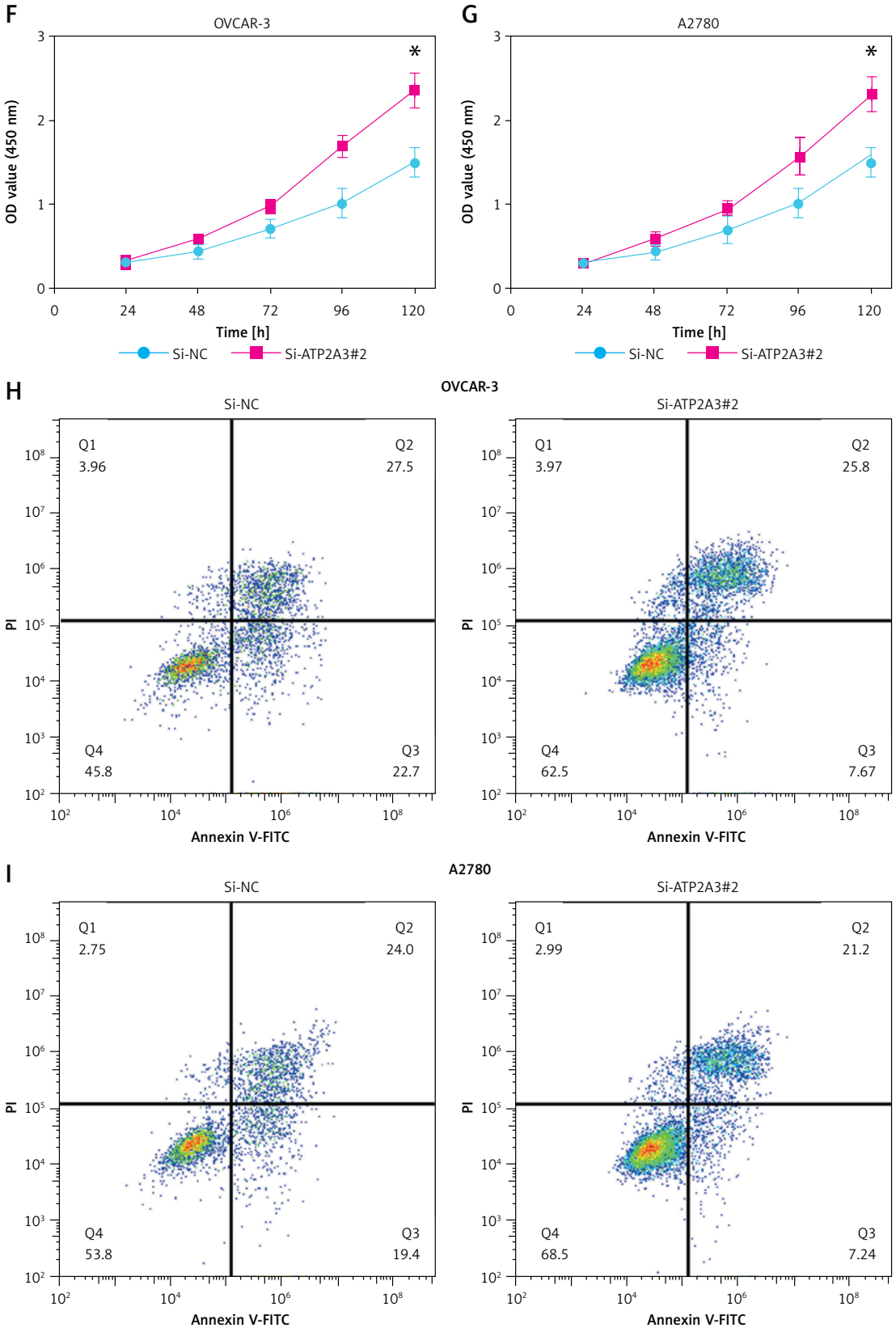


Figure 2. Cont. **F**, **G** – CCK-8 technology detected the proliferation of OVCAR-3 and A2780 cells after si-ATP2A3#2. **H**, **I** – Flow cytometry analysis depicted the apoptotic status of OVCAR-3 and A2780 cells after si-ATP2A3#2 treatment. The quadrants in the flow cytometry plot are labeled Q1, Q2, Q3, and Q4, representing early apoptosis cells, late apoptosis cells, live cells, and necrotic cells, respectively. *P < 0.05, **P < 0.01

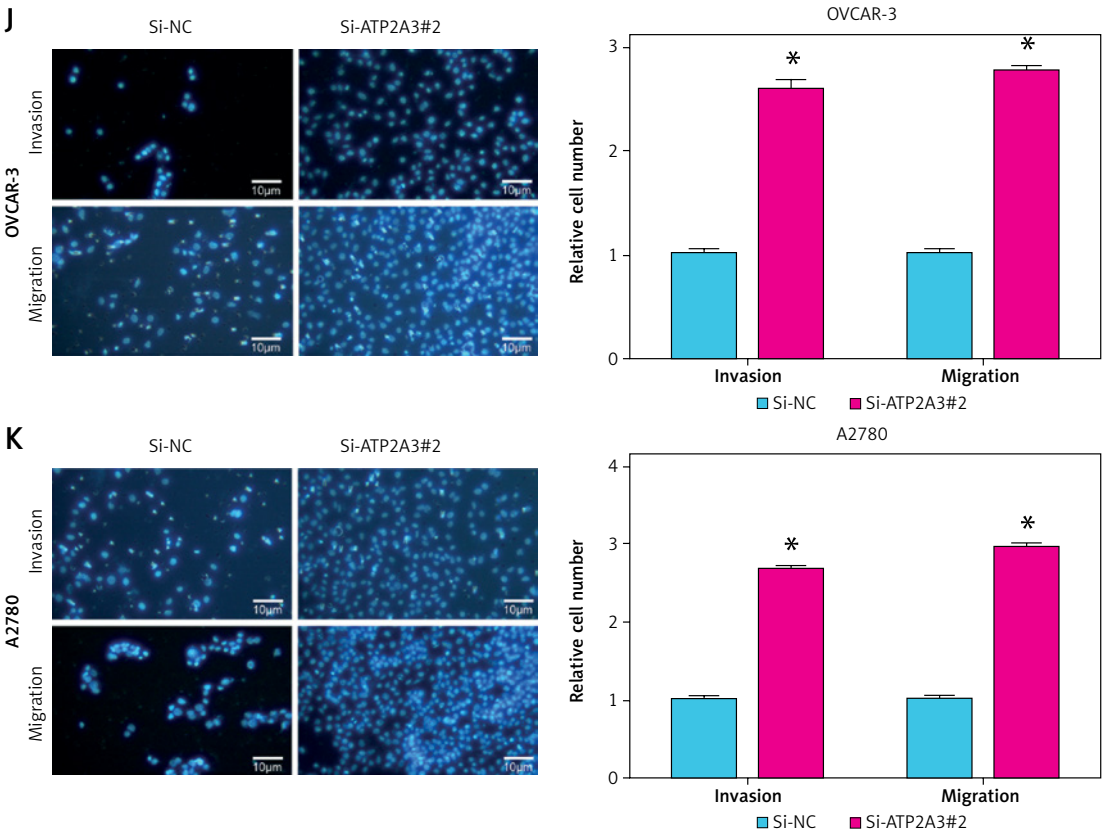


Figure 2. Cont. **J**, **K** – Transwell assays illustrated the changes in invasion and migration abilities of OVCAR-3 and A2780 cells after si-ATP2A3#2 treatment. The "10 μm" annotation in the images refers to the magnification scale bar, indicating a length of 10 micrometers at the given magnification. *P < 0.05, **P < 0.01

sessed the influence of ATP2A3 modulation on OV cell behavior. CCK-8 assays revealed that knockdown of ATP2A3, specifically using si-ATP2A3 #2, significantly augmented the proliferative capacity of OV cells, while ATP2A3 overexpression led to a marked decrease in cell proliferation (Figures 2 F, G and 3 A, B). Flow cytometry analyses indicated that compared to control cells, ATP2A3 knockdown considerably curtailed apoptosis, whereas its overexpression fostered cell apoptosis (Figures 2 H, I and 3 C, D). Furthermore, Transwell migration and invasion assays highlighted that knocking down ATP2A3 using si-ATP2A3 #2 significantly bolstered the migratory and invasive aptitude of OV cells. In contrast, ATP2A3 overexpression considerably attenuated these capacities (Figures 2 J, K and 3 E, F). These findings collectively underscore the pivotal role of ATP2A3 in modulating the aggressive behavior of OV cells, suggesting that it may serve as a promising therapeutic target.

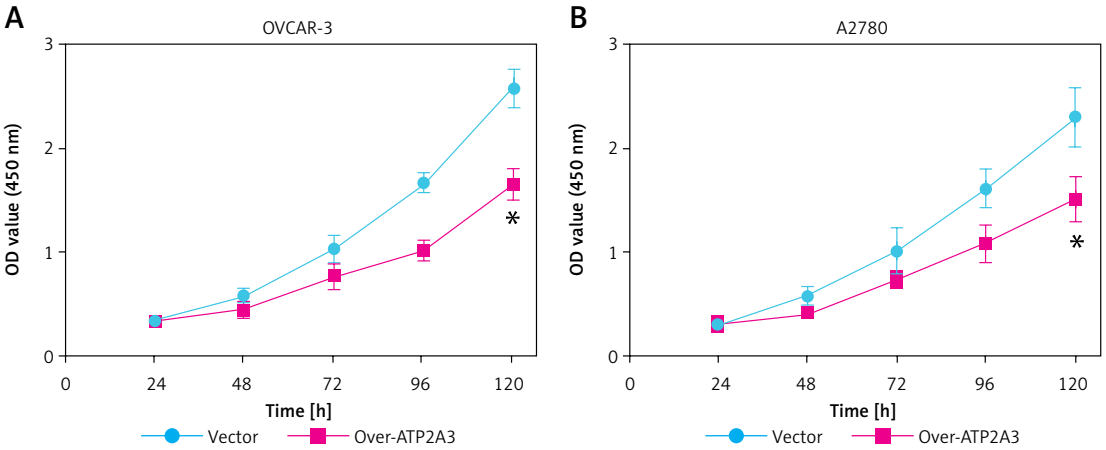


Figure 3. Effects of ATP2A3 overexpression on the behavior of OVCAR-3 and A2780 cells. **A**, **B** – Proliferation of A2780 and OVCAR-3 cells was detected by CCK-8 after ATP2A3 overexpression. *P < 0.05

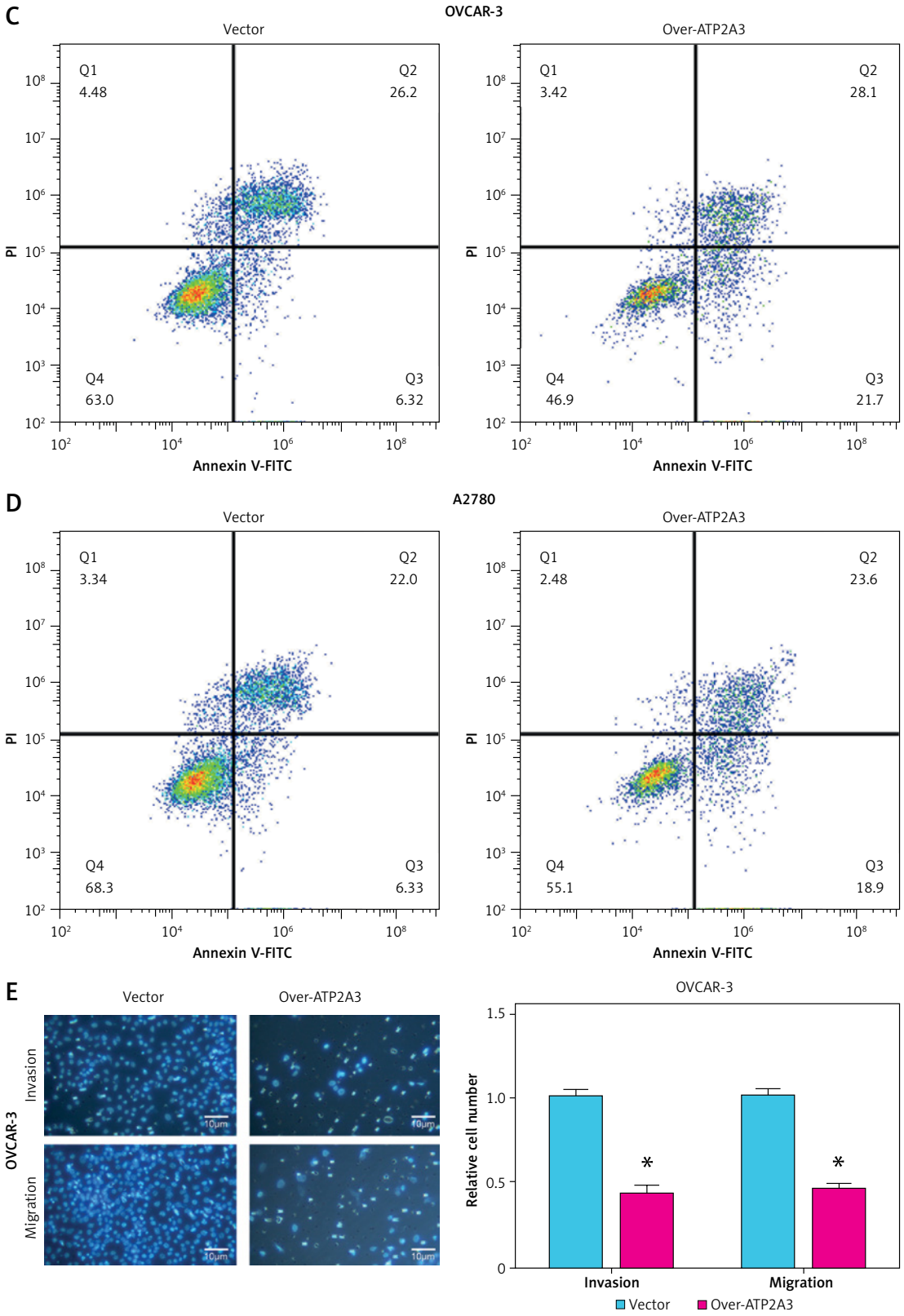


Figure 3. Cont. **C**, **D** – Flow cytometry was used to detect apoptosis of A2780 and OVCAR-3 cells after ATP2A3 overexpression (over-ATP2A3). Q1, Q2, Q3, and Q4 represent early apoptotic cells, late apoptotic cells, live cells, and necrotic cells, respectively. **E** – Transwell detection of OV cell invasion and migration changes after over-ATP2A3, and the enlarged scale bar is "10 μm". *P < 0.05

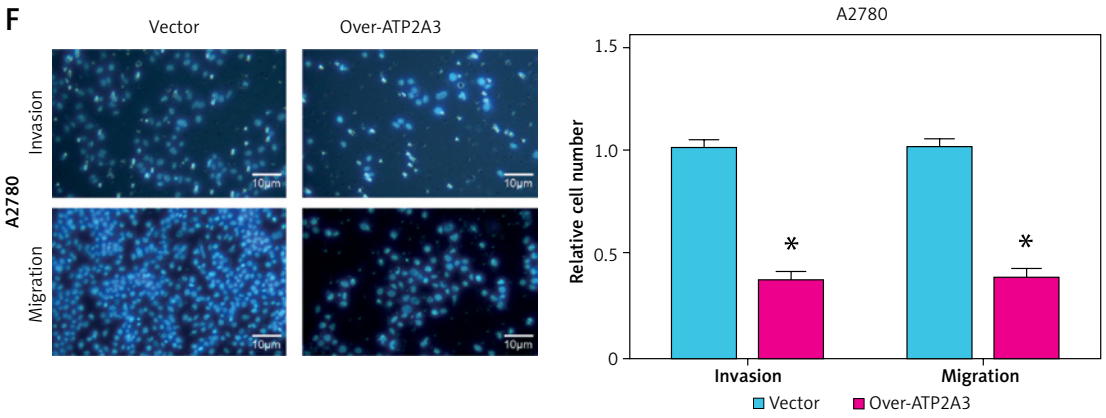


Figure 3. Cont. **F** – Transwell detection of OV cell invasion and migration changes after over-ATP2A3, and the enlarged scale bar is "10 μm". *$P < 0.05$

ATP2A3 overexpression modulates calcium-mediated kinase activities, leading to enhanced ER stress in OV cells

ATP2A3, a known regulator of calcium transport, has been shown to play an instrumental role in mediating ER stress upon aberrant expression [22]. It is noteworthy that the activity of calcium/calmodulin-dependent protein kinases (CaMK) is profoundly influenced by intracellular calcium concentrations [23, 24]. With this premise, we utilized BAPTA-AM, a chelator that rapidly sequesters free intracellular calcium [25]. The WB analysis results showed that overexpression of ATP2A3 in OV cell lines resulted in enhanced expression of ER stress biomarkers (BIP, Caspase 12, CHOP, ATF4) and ER stress-associated kinases (p-PERK, PERK, p-eIF2a, eIF2a). However, it concomitantly led to diminished protein expression of CaMK (p-CaMKIIa, CaMKIIa). Remarkably, ATP2A3 knockdown presented a reversal of these observations (Figures 4 A–C). When cells overexpressing ATP2A3 were

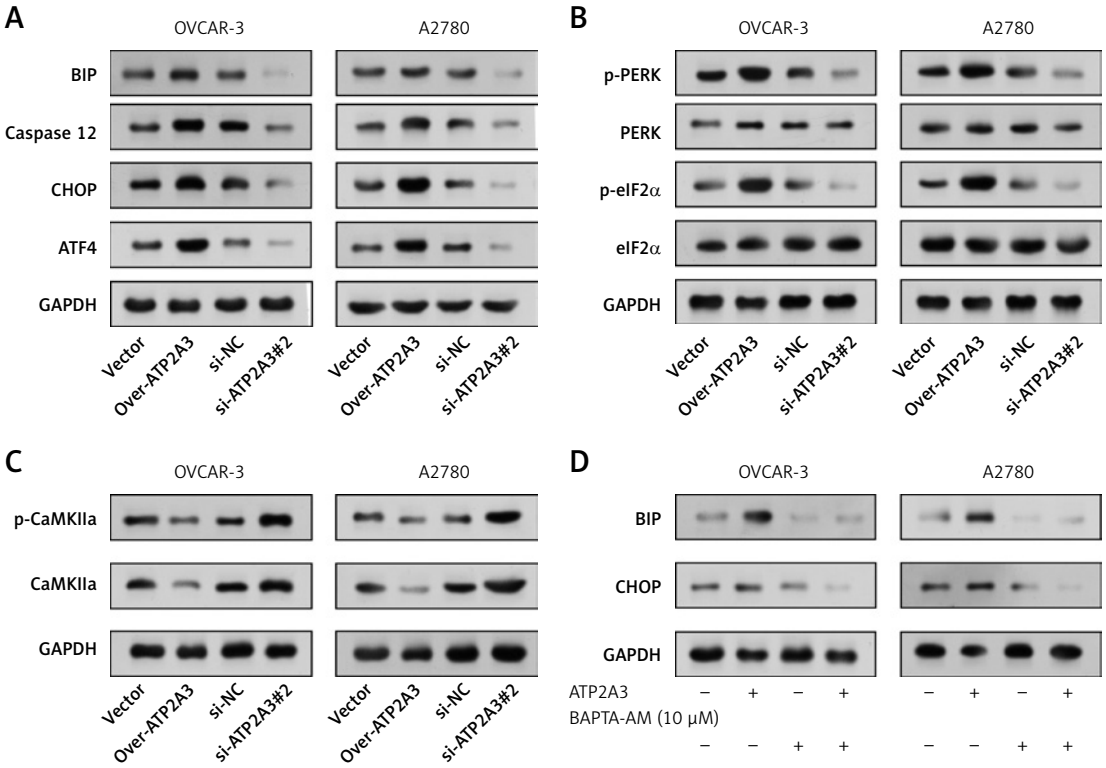


Figure 4. ATP2A3 overexpression enhances ER stress and regulates calcium-dependent kinase activity in OV cells. **A–C** – Levels of ER stress biomarkers, ER stress-associated kinases, and calcium-dependent kinase proteins were detected by WB after ATP2A3 overexpression and knockdown in OV cells. **D** – WB detection of changes in expression of ER stress biomarkers (BIP and CHOP) in OV cells overexpressing ATP2A3 and treated with a calcium chelator (BAPTA-AM). The "+" and "-" symbols denote experimental conditions in the presence or absence of a particular factor, respectively

treated with the calcium chelator BAPTA-AM, we observed a substantial reduction in the protein expression levels of BIP and CHOP (Figure 4 D). Further, WB analysis revealed that upon overexpression of ATP2A3 combined with the administration of the ER stress antagonist 4-PBA, there was a discernible decline in the expression levels of BIP and CHOP (Figure 5 A). Flow cytometry results showed that the proportion of apoptotic cells increased after ATP2A3 overexpression. Interestingly, the

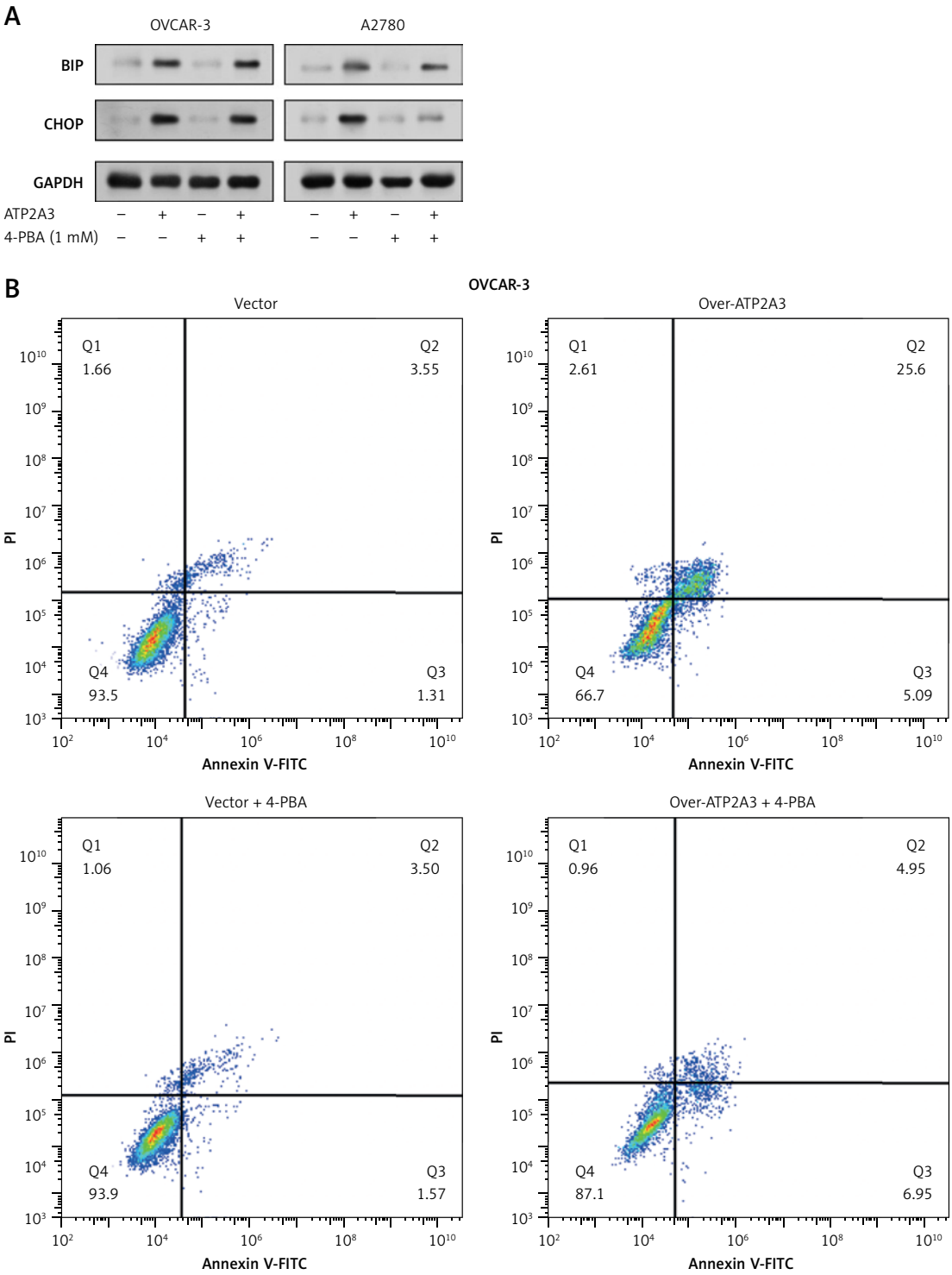


Figure 5. Regulation of ER stress biomarker expression and apoptosis in OV cell lines by overexpressed ATP2A3 combined with ER stress antagonists. **A** – Expression levels of endoplasmic reticulum stress biomarkers after over-ATP2A3+ER stress antagonist (4-PBA) in OV cell lines were measured by WB. The "+" and "-" symbols denote experimental conditions in the presence or absence of a particular factor, respectively. **B** – Flow cytometry detection of apoptosis of A2780 and OVCAR-3 cells after treatment with over-ATP2A3 combined with 4-PBA

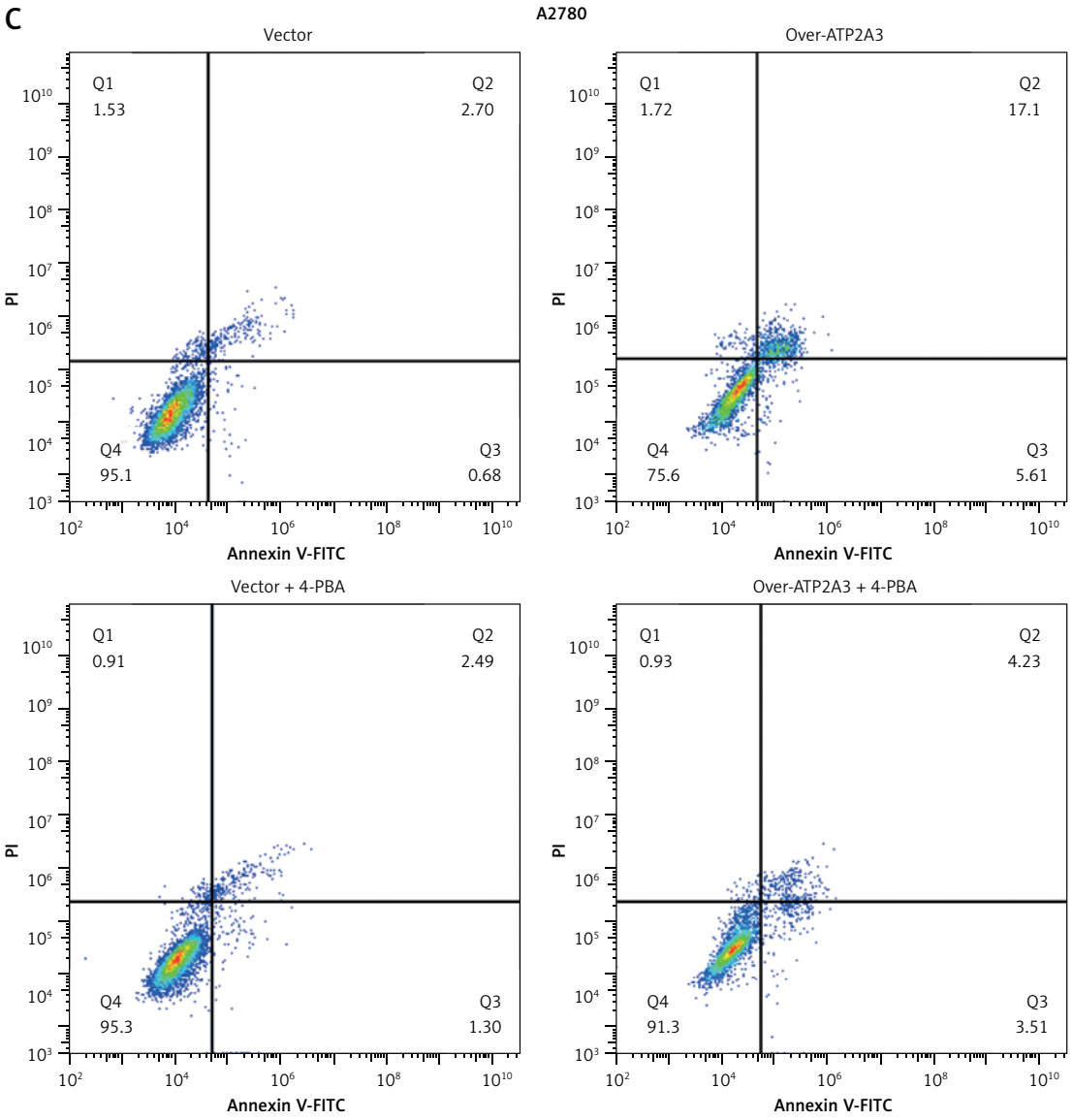


Figure 5. Cont. **C** – Flow cytometry detection of apoptosis of A2780 and OVCAR-3 cells after treatment with over-ATP2A3 combined with 4-PBA

pro-apoptotic effects of ATP2A3 were mitigated upon the addition of 4-PBA (Figures 5 B, C). Collectively, these findings underscore that ATP2A3 overexpression potentiates ER stress in OV cells, potentially through modulating intracellular calcium dynamics. Moreover, the ER stress induced by ATP2A3 can be counteracted by interventions that either sequester intracellular calcium or directly antagonize ER stress pathways.

Interaction between RXRA and ATP2A3 in OV

From the TCGA database, we identified 4,073 downregulated DEGs and 2,333 upregulated DEGs (Figure 6 A). Intriguingly, by cross-referencing TCGA-downregulated DEGs, prognostic genes, and ATP2A3 target genes, three overlapping genes were discerned: RXRA, STAT5A, and FOS (Figure 6 B). Among these three overlapping genes, for STAT5A and FOS, there is relatively scant research on RXRA in ovarian cancer. Therefore, in this study, we elected to focus on RXRA for further investigation. Analysis from the GEPIA database revealed higher expression of RXRA in OV tissues compared to their normal counterparts (Figure 6 C). The KM survival curves further elucidated that patients with high RXRA expression exhibited a greater probability of relapse-free survival (RFS) than those with low RXRA expression (Figure 6 D). Spearman correlation analysis buttressed these findings by demonstrating a significant correlation between ATP2A3 and RXRA expression (Figure 6 E). To fully understand the function of RXRA in OV, we performed qRT-PCR and WB detection. The results showed that the expression of RXRA

was increased in OVCAR-3 and A2780 cell lines after overexpression transfection (Figures 6 F, G). Moreover, upon RXRA overexpression, marked upregulation of ATP2A3 expression was observed, underscoring a potential interplay between RXRA and ATP2A3 in the context of OV (Figures 6 H, I).

RXRA regulates expression of ATP2A3 and inhibits proliferation, invasion, and migration of OV cells

We used the JASPAR database (https://jaspar.genereg.net/) to predict two potential RXRAs

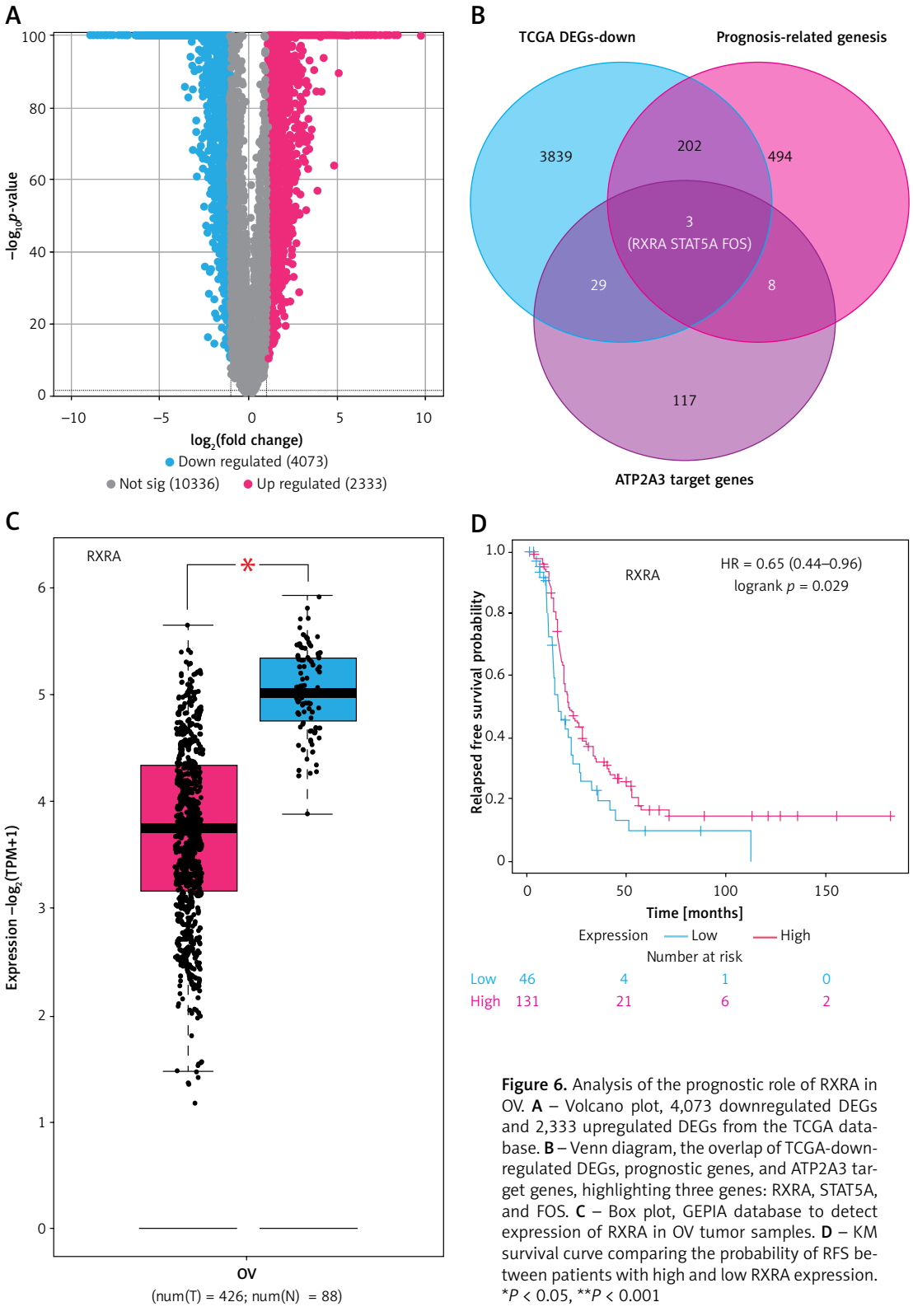


Figure 6. Analysis of the prognostic role of RXRA in OV. **A** – Volcano plot, 4,073 downregulated DEGs and 2,333 upregulated DEGs from the TCGA database. **B** – Venn diagram, the overlap of TCGA-downregulated DEGs, prognostic genes, and ATP2A3 target genes, highlighting three genes: RXRA, STAT5A, and FOS. **C** – Box plot, GEPIA database to detect expression of RXRA in OV tumor samples. **D** – KM survival curve comparing the probability of RFS between patients with high and low RXRA expression. *$P < 0.05$, **$P < 0.001$

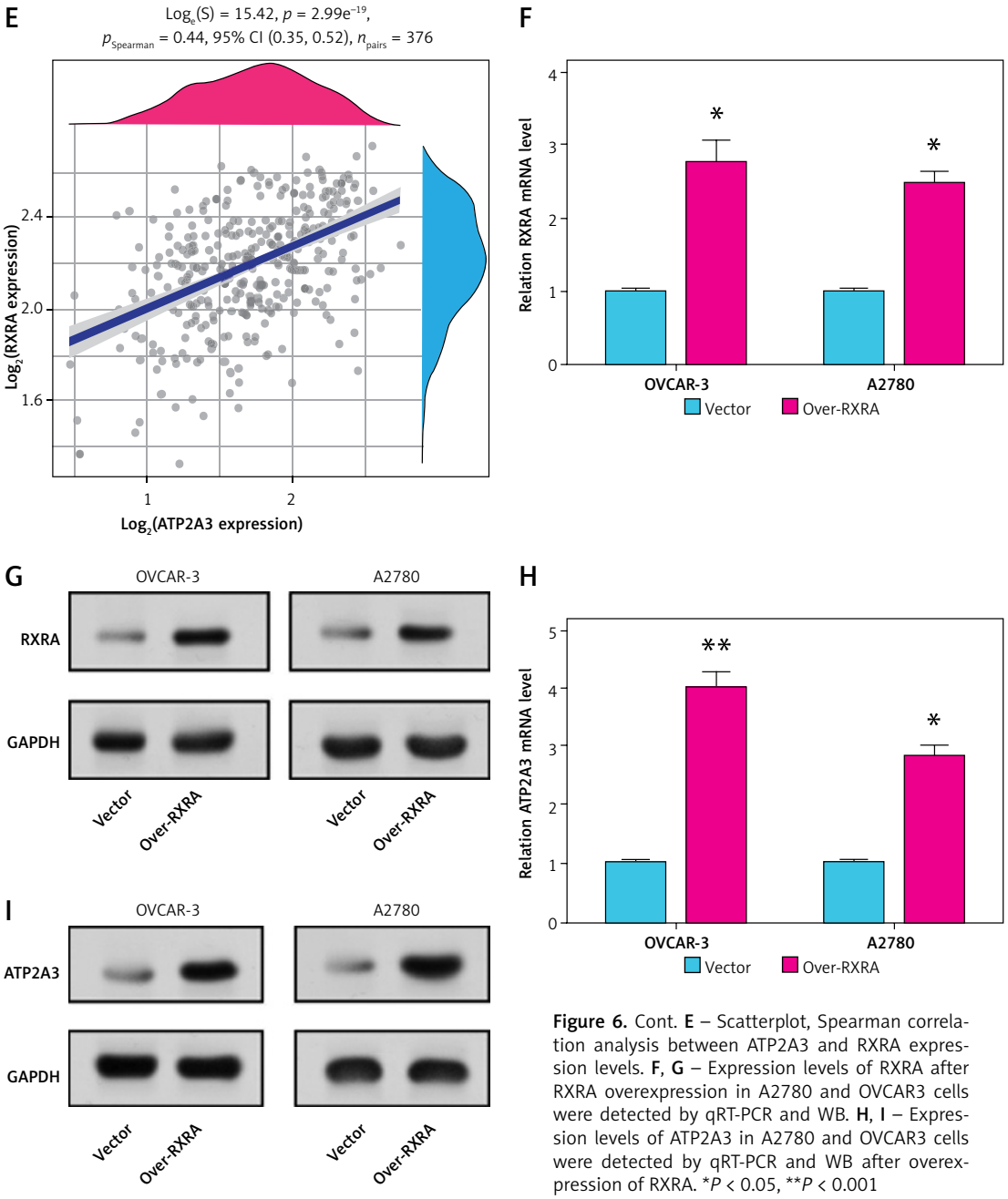


Figure 6. Cont. **E** – Scatterplot, Spearman correlation analysis between ATP2A3 and RXRA expression levels. **F**, **G** – Expression levels of RXRA after RXRA overexpression in A2780 and OVCAR3 cells were detected by qRT-PCR and WB. **H**, **I** – Expression levels of ATP2A3 in A2780 and OVCAR3 cells were detected by qRT-PCR and WB after overexpression of RXRA. *P < 0.05, **P < 0.001

bound to the ATP2A3 promoter, as shown in Figure 7 A. Then two luciferase reporter genes were constructed, and the luciferase reporter plasmids containing WT-VGLL4-S1 or WT-VGLL4-S2 sites were activated after RXRA overexpression (Figures 7 B, C). This indicated a direct interaction between RXRA and the above sites within the ATP2A3 promoter. Further validated by ChIP assays, both loci showed significant enrichment in RXRA samples, indicating a strong binding affinity of RXRA to the ATP2A3 promoter at these sites (Figure 7 D). Functionally, CCK-8 and Transwell assays showed that overexpression of RXRA significantly diminished OV cell proliferation, invasion, and migration capabilities. Intriguingly, the suppression of ATP2A3 using si-ATP2A3#2 partially counteracted the effects of RXRA overexpression on cell proliferation, invasion, and migration, hinting at a potential interplay between RXRA and ATP2A3 in modulating these cellular behaviors (Figures 7 E–H).

RXRA mediates apoptosis and ER stress in OV cells through modulation of ATP2A3 activity

In the apoptosis assay, the percentage of apoptotic cells was significantly increased after RXRA overexpression compared with the control group. However, the simultaneous knockdown of

ATP2A3#2 negated the pro-apoptotic effect induced by RXRA, resulting in a significant decrease in the apoptosis rate (Figures 8 A, B). This implies that the effect of RXRA on apoptosis may be mediated through the activity of ATP2A3. In a related assay, WB measurements revealed an elevated protein expression level of ER stress biomarkers following RXRA overexpression. Interestingly, this heightened protein expression was significantly attenuated upon ATP2A3#2 knockdown, emphasizing the interplay between RXRA and ATP2A3 in regulating ER stress responses in OV cells (Figure 8 C).

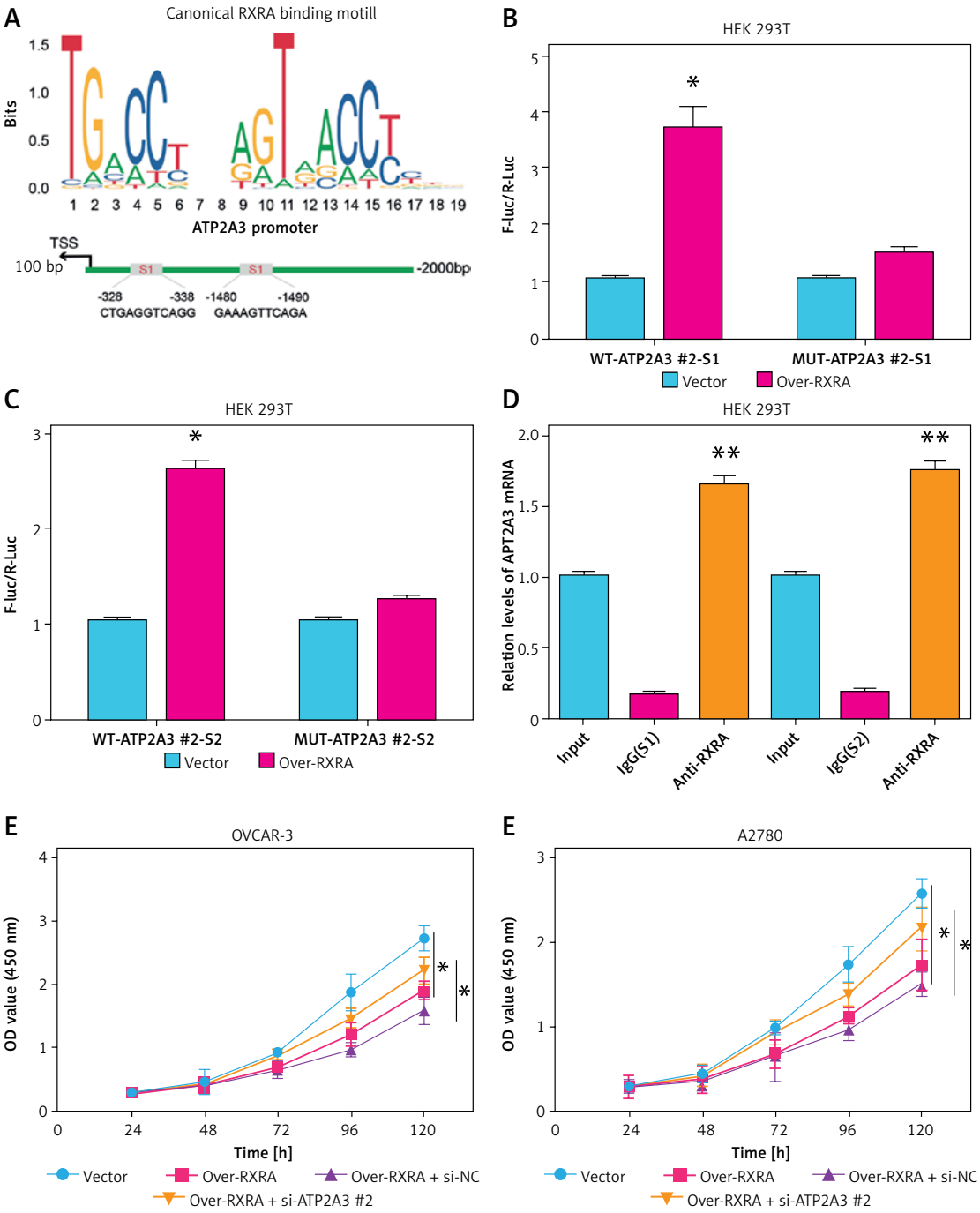
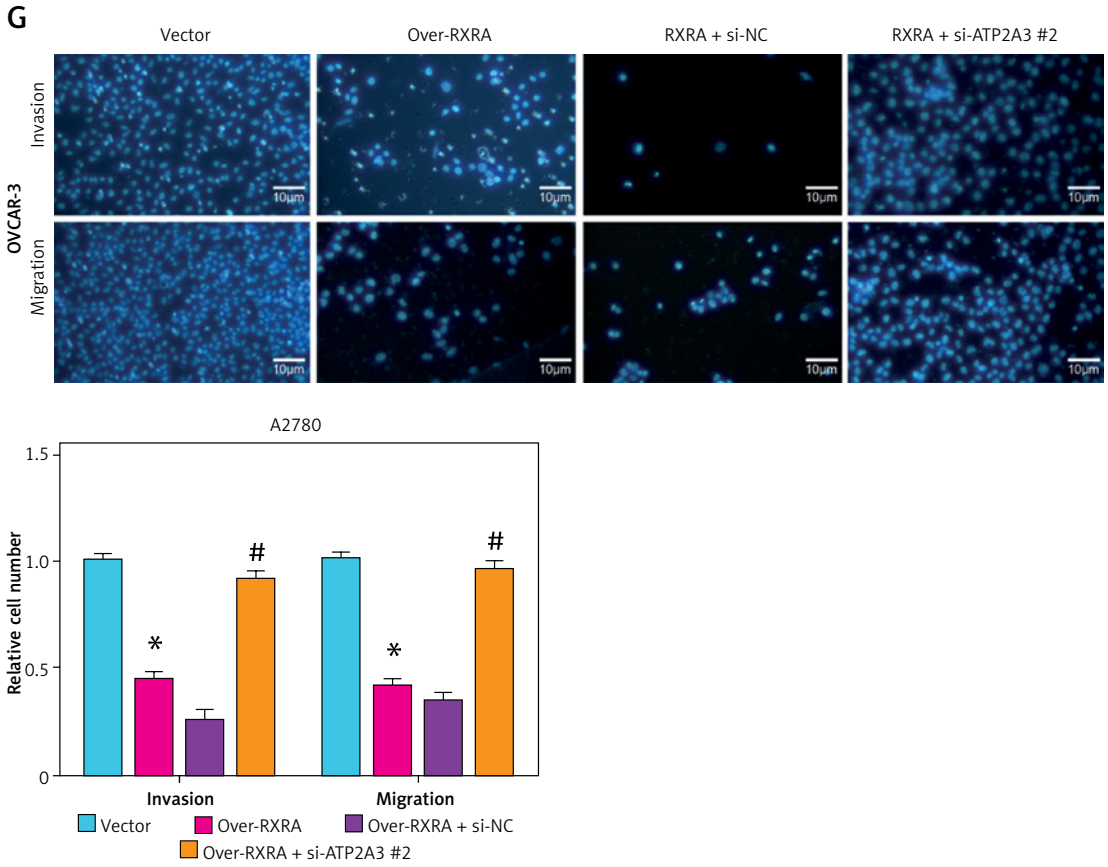


Figure 7. RXRA binds to ATP2A3, regulates OV cell proliferation, invasion, and migration. **A** – The JASPAR database was used to predict RXRA binding sites within the ATP2A3 promoter. TSS stands for transcription start site, which is the location where gene transcription starts. **B**, **C** – Two luciferase reporter genes were constructed to measure the luciferase activity at the RXRA binding site. **D** – ChIP assay detects significant binding of RXRA to predicted binding sites on the ATP2A3 promoter. **E**, **F** – CCK-8 assays for the effects of RXRA overexpression and ATP2A3 inhibition on OV cell proliferation. *$P < 0.05$, **$P < 0.01$



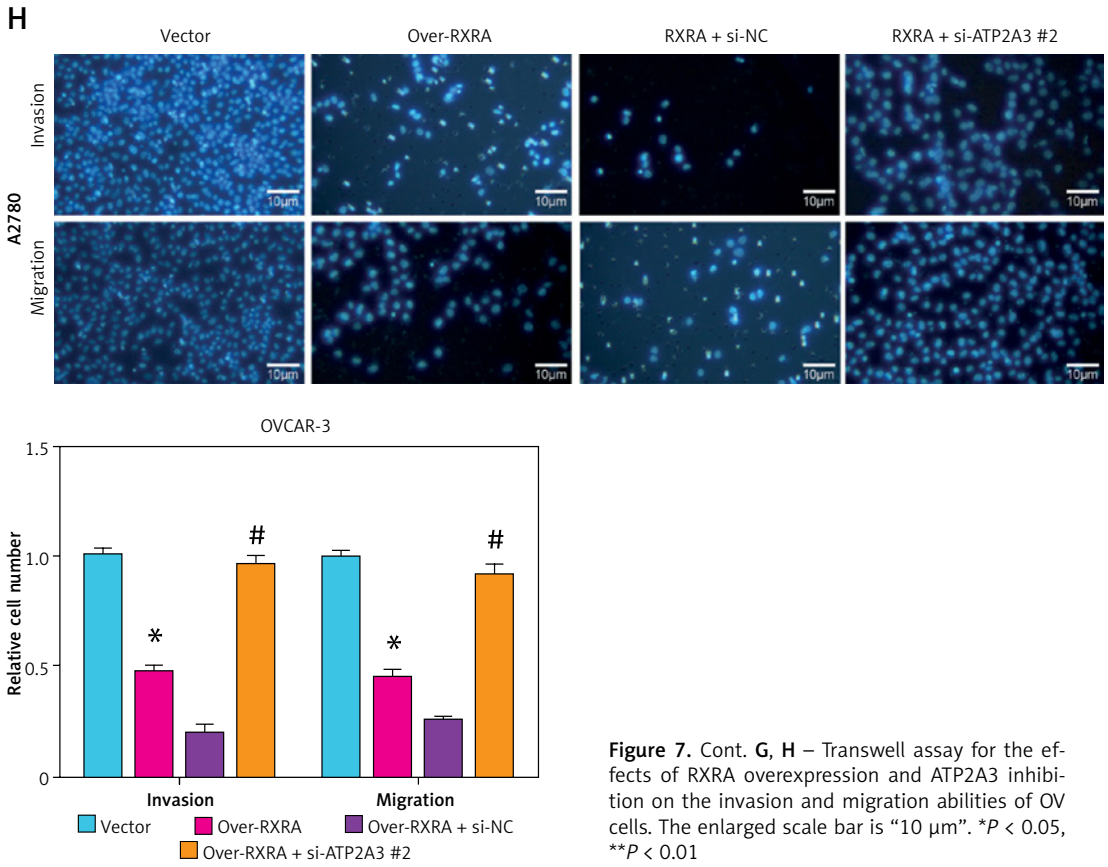


Figure 7. Cont. **G**, **H** – Transwell assay for the effects of RXRA overexpression and ATP2A3 inhibition on the invasion and migration abilities of OV cells. The enlarged scale bar is "10 μm". *P < 0.05, **P < 0.01

Discussion

In the realm of cellular physiology, the role of intracellular calcium ions (Ca^{2+}) as a crucial mediator cannot be overlooked. Its modulation plays pivotal roles in both physiological and pathological cellular signaling, influencing cellular fate, growth, and survival [26, 27]. Disturbances in Ca^{2+} homeostasis have been identified in various pathological conditions, including tumorigenesis [28]. Particularly in OV, interventions targeting calcium channels have demonstrated therapeutic promise. Studies have shown that blocking T-type Ca^{2+} channels can inhibit the proliferation of human OV cells [29]. Another study showed that inhibition of the Ca^{2+}-activated chloride channel ANO1 inhibits OV by inactivating PI3K/Akt signaling [30]. Our study delved deeper into the role of the ATP2A gene family and, notably, its interaction with RXRA. The ATP2A genes – ATP2A1, ATP2A2, and ATP2A3 – are encoded on distinct chromosomes but collectively influence the functions of the sarco/endoplasmic reticulum Ca^{2+} ATPase pump (SERCA), a key determinant of Ca^{2+} homeostasis [31]. This study underscores the significance of ATP2A3 and its transcriptional regulator RXRA in OV, expanding our understanding of how cellular calcium regulation can be targeted for therapeutic interventions.

The ATP2A gene family, particularly ATP2A3, has garnered attention due to its pivotal role in

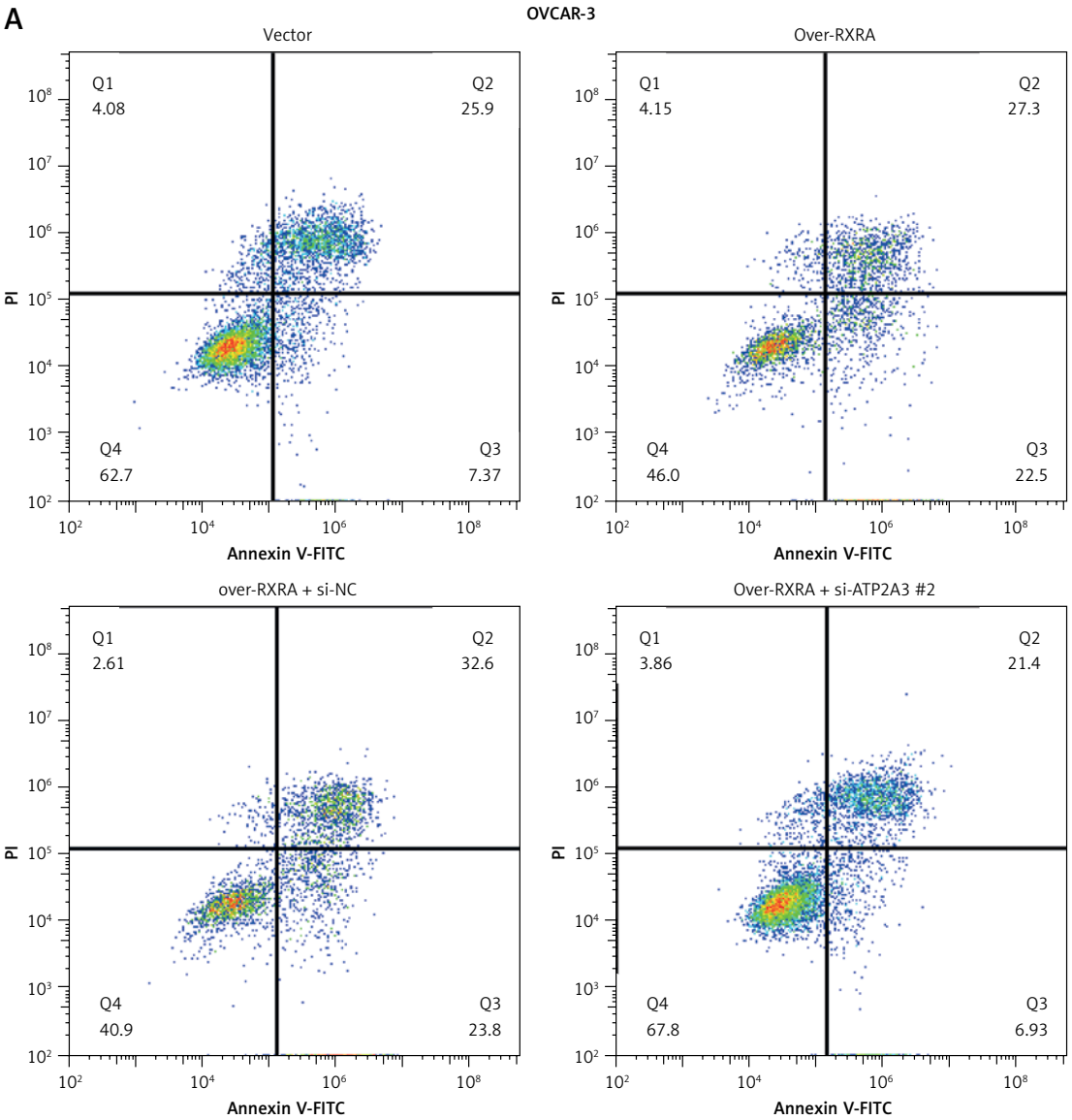
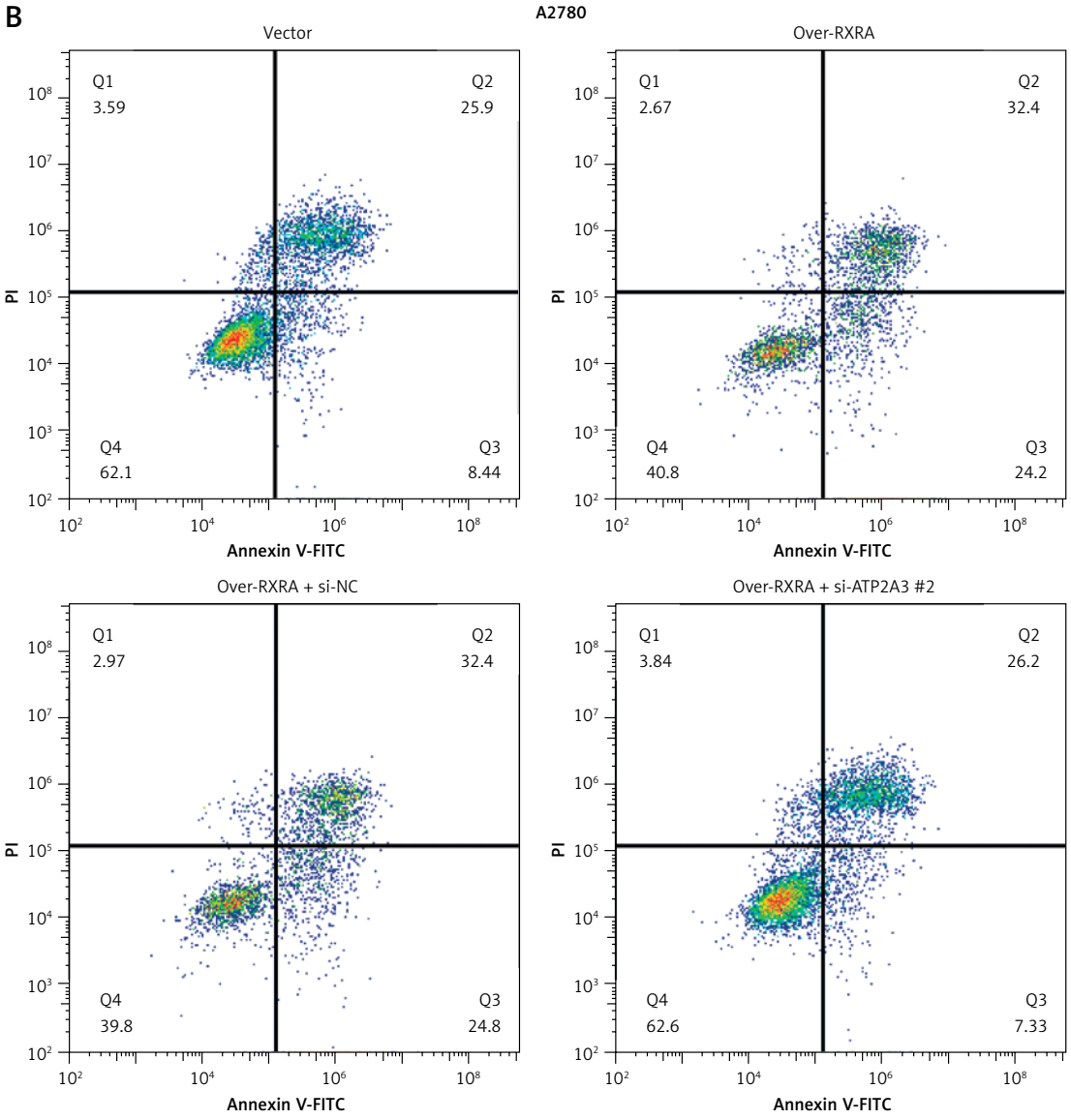


Figure 8. Effects of RXRA overexpression and ATP2A3 knockdown on apoptosis and endoplasmic reticulum stress in OV cells. **A**, **B** – Flow cytometry to measure apoptosis of OV cells after Vector, over-RXRA, over-RXRA + si-NC, over-RXRA + si-ATP2A3 #2 in OVCAR-3 and A2780 cell lines. Q1: early apoptotic cells, Q2: late apoptotic cells, Q3: live cells, Q4: necrotic cells



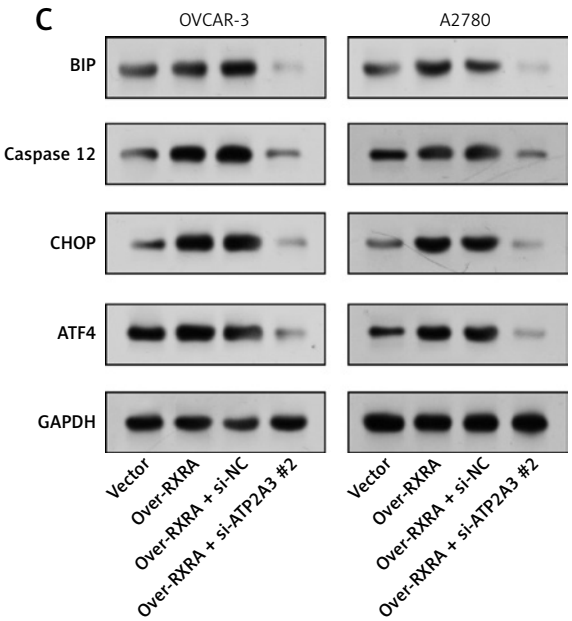


Figure 8. Cont. **C** – WB detection of expression levels of ER stress biomarkers after over-RXRA, over-RXRA + si-NC, over-RXRA + si-ATP2A3 #2 treatment in OVCAR-3 and A2780 lines

intracellular calcium regulation, a process integral to various cellular functions. ATP2A3 encodes one of the SERCA Ca $^{(2+)}$-ATPases, an enzyme integral to calcium transfer from the cytosol to the sarcoplasmic reticulum, modulating calcium sequestration during muscle excitation [32, 33]. Previous studies have connected ATP2A3 to the pathogenesis of cancers, including breast and gastric cancers. For instance, Zarain-Herzberg *et al.* found that ATP2A3 was an important participant in the anti-cancer activity of resveratrol in breast cancer cells [34]. Another study showed that ATP2A3 regulates cytoplasmic Ca^{2+} concentration in KATO-III gastric cancer cells, and its expression is influenced by specific therapy and epigenetic modifications, which may indicate the prognosis of patients with gastric cancer [35]. However, its relationship with OV remained relatively unexplored. In our study, expression analyses highlighted a varied expression pattern of the ATP2A genes in OV samples. Notably, ATP2A3 showed consistent associations with improved survival outcomes in OV, corroborating its potential protective role. Additionally, the reduced ATP2A3 protein levels in primary OV samples compared to normal ones underscore its potential clinical significance. This finding, coupled with the significant variations in ATP2A3 expression across different clinicopathological parameters, suggests a multifaceted role of ATP2A3 in OV progression and its potential as a therapeutic target.

In our pursuit to delineate the cellular implications of ATP2A3 dysregulation in OV, we executed in vitro experiments that shed light on this intricate interplay. Notably, ATP2A3 was markedly downregulated in key OV cell lines, especially OVCAR-3 and A2780, highlighting its potential tumor-suppressive role. The differential expression of ATP2A3 exerted dichotomous effects on OV cell proliferation, apoptosis, migration, and invasion, underscoring its pivotal role in tempering the malignancy of OV cells and spotlighting its therapeutic promise. Probing deeper into the molecular intricacies, we observed that overexpression of ATP2A3 in OV cell lines robustly augmented the expression of ER stress biomarkers and ER stress-associated kinases, while concurrently diminishing the expression of CaMK. Remarkably, knocking down ATP2A3 reversed these effects. Moreover, the introduction of BAPTA-AM and the ER stress antagonist 4-PBA mitigated the effects of ATP2A3 overexpression. Such observations suggest that ATP2A3 may modulate the expression and apoptosis of OV cells by mediating ER stress, potentially influencing Ca^{2+} homeostasis.

In our bioinformatics analysis, we screened RXRA from both TCGA and HTFtarget databases. Retinoid X receptor alpha (RXRA), a prominent member of the nuclear hormone receptor superfamily, encompasses ligand-activated transcription factors pivotal in myriad biological processes [36]. RXRA, ubiquitously expressed across various tissues and organs, transcends its canonical role in the retinoid signaling pathway, assuming multifaceted functionalities. Aberrant RXRA expression or activity has been implicated in a plethora of pathological conditions, spanning from malignancies to metabolic dysfunctions and neurodegenerative disorders [37, 38]. Notably, it has been documented that the transcription factor RXRA-mediated regulation of PLD1 curtails the long non-coding RNA LINC00511, thus facilitating autophagy and apoptosis in cervical cancer cells [39]. Concurrently, interactions between miR-191 and RXRA have been demonstrated to exert specific influences on prostate cancer dynamics [40]. Intriguingly, our findings revealed diminished RXRA expression in tumor specimens relative to their normal counterparts. Moreover, elevated RXRA expression portended enhanced recurrence-free survival in OV. Significantly, a positive correlation was found between ATP2A3 expression and RXRA. Further analysis of the data hinted at a potential promoter synergy between RXRA and ATP2A3. The upregulation of RXRA significantly enhanced the luciferase activity in the binding region of ATP2A3, suggesting that RXRA plays a major role in regulating ATP2A3 transcription. On this basis, our in vitro evaluations consistently showed that the surge of RXRA expression favored apoptosis of OV cells, hindered their growth, invasion, and migration, and increased the expression level of ER stress markers. Interestingly, silencing ATP2A3 appears to partially neutralize the cellular effects of RXRA upregulation. This finding highlights the critical role of RXRA as a tumor suppressor and its far-reaching impact on coordinating endoplasmic reticulum stress dynamics. Given these findings, deeper exploration of the molecular complexity of RXRA may open up new avenues for the treatment of OV and other related malignancies.

While our *in vitro* analyses have yielded significant insights into the role of ATP2A3 in the regulation of cellular processes such as proliferation, apoptosis, migration, and invasion in ovarian cancer cells, the extrapolation of these findings to the intricate tumor microenvironment *in vivo* has not been established. We recognize that the absence of in vivo confirmation represents a limitation in our study, potentially affecting the comprehensive assessment of ATP2A3's contribution to tumorigenesis and metastasis. To mitigate this, we are committed to integrating *in vivo* model systems in our forthcoming research. This approach will allow us to rigorously validate the effects of ATP2A3 modulation on ovarian cancer progression and to

explore its therapeutic potential. By extending our investigations in this direction, we aim to deepen our comprehension of ATP2A3's role in ovarian cancer and to contribute to the formulation of more efficacious clinical interventions for patients afflicted with this malignancy.

In conclusion, our comprehensive *in vitro* cellular assays combined with bioinformatics analysis demonstrated that the suppression of ATP2A3 and RXRA augments the proliferation, migration, and invasion capacities of OV cells. Conversely, their upregulation fosters apoptosis while concurrently diminishing cell proliferation and migration. A noteworthy regulatory interplay was identified between RXRA and ATP2A3, with RXRA acting as an upstream enhancer of ATP2A3 expression. The elevated expression of both RXRA and ATP2A3 amplifies the levels of endoplasmic reticulum stress markers. This suggests a pivotal role in modulating OV cell dynamics and apoptosis, potentially through the mediation of Ca^{2+} homeostasis via the ER stress response. To conclude, our findings shed light on the intricate interrelationship and mechanistic underpinnings of ATP2A3 and RXRA in the trajectory and prognosis of OV, offering invaluable insights for therapeutic interventions and prognostic evaluations in OV.

Acknowledgments

Lina Zhao and Lidong Zeng contributed equally.

Funding

No external funding.

Conflict of interest

The authors declare no conflict of interest.

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