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ORIGINAL RESEARCH

Investigation of the Immunoexpression of SVIP and UPR Pathway Proteins in Ovarian Adenocarcinoma Cell Line OVCAR-3

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ABSTRACT

Ovarian cancer is the deadliest gynecological cancer. The endoplasmic reticulum (ER), a vital cell organelle, is involved in the folding, synthesis, and modification of a wide range of soluble and insoluble proteins. ER stress initiates the unfolded protein response (UPR), an evolutionary conserved cell stress mechanism. The UPR is mediated by three ER transmembrane sensors: IRE1, ATF6, and PERK. An inhibitor of ERAD is a small VCP/p97-interacting protein (SVIP). The study aimed to investigate the relationship between SVIP and the ER stress protein markers in the human ovarian cancer cell line OVCAR-3. The SVIP and GRP78, PERK, ATF4 immunoexpression levels were analyzed. Furthermore, employing immunofluorescence, the colocalization of three ER sensors and SVIP was ascertained. The immunoexpression of SVIP and GRP78, ATF4, and PERK were shown in the OVCAR-3 cell line. Additionally, immunofluorescence results showed the colocalization of SVIP and UPR-related proteins in the cytoplasm of OVCAR-3 cells. In conclusion, we demonstrated the cellular localization of SVIP and the proteins involved in the UPR pathway. However, further studies are needed to determine the relation between SVIP and these proteins in cancer cells.

Keywords: Endoplasmic reticulum (ER) stress. Immunofluorescence. Ovarian adenocarcinoma cell line (OVCAR-3). Small VCP-interacting protein (SVIP). Unfolded protein response (UPR).

Ovcar-3 Ovarian Adenokarsinoma Hücre Hattında SVIP ve UPR Proteinlerinin İmmünoekspresyonunun Araştırılması

ÖZET

En ölümcül jinekolojik kanser yumurtalık kanseridir. Hayati bir hücre organeli olan endoplazmik retikulum (ER), çok çeşitli çözünür ve çözünmez proteinlerin katlanması, sentezi ve modifikasyonunda rol oynar. ER stresi, evrimsel süreçte korunmuş bir hücre stres mekanizması olan katlanmamış protein yanıtını (UPR) başlatır. UPR'a, üç ER transmembran sensörü aracılık eder: IRE1, ATF6 ve PERK. ERAD inhibitörü küçük VCP/p97 ile etkileşen proteindir (SVIP). Çalışmanın amacı, Ovcar-3 insan ovarian adenokarsinoma hücre hattında SVIP ile ER stres protein belirteçleri arasındaki ilişkiyi araştırmaktır. SVIP ve GRP78, PERK, ATF4 immünoekspresyon düzeyleri analiz edildi. Ayrıca, immünofloresan kullanılarak, üç ER sensörünün ve SVIP'in kolokalizasyonu belirlendi. SVIP ve GRP78, ATF4 ve PERK'nin immünoekspresyonu OVCAR-3 hücre hattında gösterildi. Ek olarak, immünofloresan sonuçları OVCAR-3 hücrelerinin sitoplazmasında SVIP ve UPR ile ilişkili proteinlerin kolokalizasyonunu gösterdi. Sonuç olarak, SVIP'nin hücresel lokalizasyonunu ve UPR yolunda yer alan proteinleri gösterdik ancak kanser hücrelerinde SVIP ile bu proteinler arasındaki ilişkiyi belirlemek için daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: Endoplazmik retikulum (ER) stresi. İmmünofloresan, Ovarian adenokarsinoma hücre hattı (OVCAR-3). Küçük VCP ile etkileşen protein (SVIP). Katlanmamış protein yanıtı (UPR).

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Gynecologic malignancies commonly result in cancer mortality from ovarian cancer (OC) globally[1,2]. The diverse nature of this malignancy, its late presentation, and the absence of appropriate screening may all contribute to its high cure rate and frequent diagnosis in advanced stages[2]. Family history, early menarche, obesity, reproductive treatments, diabetes, alcohol use, aging, and smoking are the primary risk factors for OC[3-5]. Patients who have been diagnosed with a medical condition typically receive chemotherapy as a second line of treatment after surgery. Most patients with advanced OC are likely to develop resistance to chemotherapy, which is the primary reason for treatment failure[6].

The endoplasmic reticulum (ER) is an internal organelle that plays a role in maintaining cellular homeostasis, the delicate balance between health and sickness, and the synthesis and folding of cellular proteins. Only correctly folded proteins are allowed to enter their cell compartment, as the ER ensures that these processes occur. It is also in charge of the synthesis, maturation, folding, quality control, and destruction of transmembrane and secreted proteins. When misfolded or unfolded proteins build up in the ER lumen, a particular ER stress pathway called the unfolded protein response (UPR) is triggered. When cellular demands ER's ability to fold proteins correctly, or when there is insufficient cellular energy available to fold proteins produced into the ER, incorrectly folded proteins can accumulate[7]. Through a variety of mechanisms, the UPR aids in the restoration of homeostasis in cells: (i) by decreasing the rate of protein synthesis; (ii) by enhancing the ER's ability to fold proteins and remove unfolded or misfolded proteins; and (iii) by turning on chaperones and heat shock proteins to promote the accumulation of misfolded proteins and cell cycle arrest[8].

The activating transcription factor 6 (ATF6), protein kinase RNA-activated (PKR)-like ER kinase (PERK), and inositol-requiring enzyme 1 (IRE1) are the three main sensors that regulate the UPR. These stress sensors are kept in an inactive state and are bound by the ER chaperone GRP78/BiP[9,10]. When misfolded proteins are kept in the ER, they cause the production of ER-resident chaperones, activate GRP78/BiP, and temporarily reduce protein synthesis. The UPR is regarded as an adaptive and cytoprotective activity because it restores ER capacity by rebalancing protein load and folding[11].

When PERK is activated, eIF2α is serine-phosphorylated and its translational activity is inhibited, which prevents protein synthesis[12]. This mechanism facilitates the production of Ca-independent transcription by reducing protein overload and the buildup of misfolded proteins under ER stress[13]. In order to decrease total protein translation, PERK preferentially increases ATF4 by phosphorylating eukaryotic translational initiation factor 2 (eIF2)[14].

The maturation and degradation of UPR are caused by the endoribonuclease activity of IRE1, the third regulator of UPR is the creation of XBP1s, a splicing variation of XBP1. This transcriptional factor reduces ER stress by activating a number of downstream genes involved in protein secretion, maturation, and degradation[15].

Numerous investigations have shown that carcinomas of the breast, stomach, colon, esophagus, lung, prostate, pancreatic, and liver, as well as leukemia, lymphoma, and myeloma, are all highly activated forms of the UPR (IRE1, PERK, and ATF6)[16].

Cellular viability depends on a cell's capacity to maintain quality control over misfolded proteins[17]. Misfolded protein buildup has been observed in various disorders and is frequently harmful to cells[18].

Cells activate ERAD when the ER quality control system is unable to refold folding intermediates and misfolded proteins. ERAD is a secondary defense mechanism that, by retro-transporting misfolded proteins from the ER into the cytoplasm, where they are ubiquitinated for proteasomal destruction[19,20], preserves homeostasis in the Golgi secretory pathway[21]. ERAD necessitates ubiquitination, coordinated extraction through pore proteins in the ER membrane, and proteasomal breakdown. One of the essential elements of protein degradation in ERAD is ERAD E3 ligase gp78[22].

As a member of the AAA (ATPase involved with diverse cellular processes) ATPase family, p97/VCP interacts with several partners and protein cofactors, including gp78, to facilitate the degradation of proteins. The binding of p97/VCP to polyubiquitinated proteins is improved by the interaction between p97/VCP and gp78[23, 24]. Through its VCP-interacting motif (VIM), p97/ VCP interacts with gp78 E3 ligase[25, 26].

The same VIM domain is found in a small p97/VCP-interacting protein (SVIP). With two possible coiled-coil regions, SVIP consists of 76 amino acids[27]. To control VCP function, SVIP may compete with E3 ligase binding to p97/VCP because it shares the VIM motif with gp78[28, 29]. The creation of vacuoles, which may result from an accumulation of misfolded proteins when SVIP is overexpressed, has demonstrated the negative regulatory role of SVIP in ERAD[30]. SVIP's ability to inhibit ERAD is diminished under ER stress due to the downregulation of the SVIP protein. On the other hand, extended ER stress causes misfolded protein to accumulate in the ER and dramatically increases SVIP[31].

Therefore, the study aimed to investigate the relationship between SVIP and the ER stress protein markers in the ovarian cancer cell line OVCAR-3.

Material and Method

Cell Culture

The OVCAR-3 human ovarian adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, HTB-161). OVCAR-3 cells were cultured in RPMI-1640 medium (Gibco, Germany) supplemented with 10% fetal bovine serum (Capricorn Scientific, Germany), 1% penicillin-streptomycin (Capricorn Scientific, Germany). The cells were maintained in a 5% CO2 incubator at 37°C. The cells were resuspended in culture media following the addition of RPMI-1640 to inactivate the trypsin and

the harvesting of semi-confluent cells from flasks containing 0.25% trypsin (Gibco, USA).

Hematoxylin and Eosin staining of cells

OVCAR-3 cells were prepared for morphological examination. The confluent cells were plated in a six-well plate comprising coverslips within each well and incubated for 24 hours. The coverslip-growing cells were applied with hematoxylin and eosin staining. The coverslips were then mounted on a glass slide and examined under the light microscope (Olympus BX43).

Single and double Immunofluorescence

A single-label immunofluorescence staining procedure was used. After Phosphate-buffered saline (PBS) cleansing, cells were treated with primary antibodies SVIP (1:50; HPA039807; Sigma), PERK (1:200; sc-377400; Santa Cruz), ATF4 (1:200, sc-390063; Santa Cruz), and GRP78 (1:200; 11587-1-AP; Proteintech) for an overnight period at 4 °C. Texas red conjugated anti-mouse secondary antibody (1:450; ab6787; Abcam), and FITC-conjugated antirabbit secondary antibody (1:450; ab6717; Abcam) were applied to the coverslips for one hour.

To demonstrate the proteins colocalized, double immunofluorescence staining was applied. The OVCAR-3 cells were seeded in 12-well plates (Thermo Scientific) on glass coverslips (NEST Scientific). Then, the cells were fixed in 4 % formaldehyde for 30 min, After three washes with PBS, the coverslips were incubated in a blocking solution (Protein Block, Abcam, ab93697). Primary antibodies (mouse anti-ATF4, rabbit anti-SVIP, mouse anti-PERK, and rabbit anti-GRP78) were simultaneously performed and incubated overnight at 4 °C. After three washes with PBS, fluorescence-conjugated anti-mouse and anti-rabbit secondary antibodies were applied to the coverslips and incubated for 1 hour at room temperature. All samples were covered with a DAPI-containing mounting medium (Abcam, ab104139), and the fluorescent images were analyzed under a fluorescent microscope (Olympus BX53).

Results

Cell morphology in OVCAR-3 cancer cell line

The morphology of living OVCAR-3 human cancer cells was analyzed under an inverted microscope (Olympus IX73) (Figure 1A) Also, the slides were stained with H&E and were photographed under the light microscope (Olympus BX43) (Figure 1B). OVCAR-3 cells showed epithelial-like morphology. The cells exhibited a tendency to growth in aggregates.

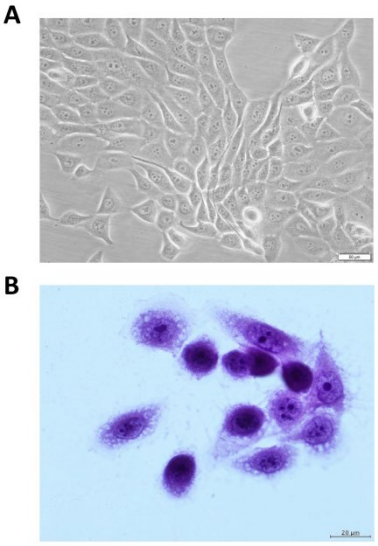

Figure 1.
Cellular morphology of ovarian cancer cell line OVCAR-3. Morphology of living OVCAR-3 cells (A). Hematoxylin and eosin (H&E) staining of OVCAR-3 cells (B). OVCAR-3 cells showed epithelial morphology. Scale bars: 50 µm (A), 20 µm (B).

SVIP, ATF4, PERK, and GRP78 are present in the human OVCAR-3 cell line

The presence of SVIP, ATF4, PERK, and GRP78 in the OVCAR-3 cell line was determined by immunofluorescence. The OVCAR-3 cells' cellular localizations and protein expressions of SVIP and ATF4, PERK, GRP78 were displayed in Figure 2. In the ovarian cancer cells' cytoplasm SVIP, ATF4, PERK, and GRP78 were visible.

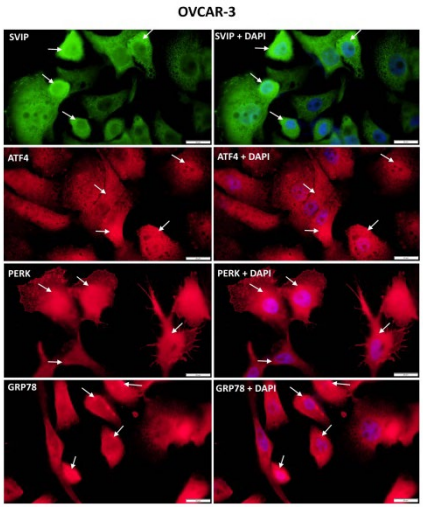

Figure 2.
Expressions of SVIP, ATF4, PERK, and GRP78 in OVCAR-3 cells. Cellular localization of SVIP, ATF4, PERK, and GRP78 proteins was detected in the cytoplasm (arrow) of OVCAR-3 cells. Scale bars: 20 µm.

Coexpression of SVIP and UPR markers in OVCAR-3 cells

Because SVIP showed similar expressions with ATF4, PERK and GRP78 colocalizations of these proteins were analysed by double-immunofluorescence, with an expectation to detect the areas where their localizations were overlapping. When OVCAR-3 was examined, it was seen that colocalized areas of SVIP and UPS proteins were determined mostly around the nucleus and in the cytoplasm. (Figure 3).

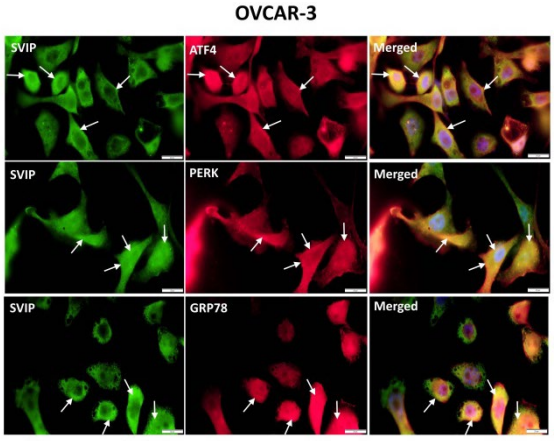

Figure 3.
Coexpression of SVIP and UPR markers (ATF4, PERK, GRP78) in OVCAR-3 cells. Detection of SVIP (green), ATF4 (red), PERK (red), GRP78 (red), and double (yellow) positive cells on OVCAR-3. Merging of nuclear 4',6-diamidino-2-phenylindole (DAPI) staining (blue) is also observed in colocalization. Scale bars: 20 μm.

Discussion and Conclusion

Ovarian cancer is the fifth most dangerous disease in women. Although medical detection and therapy have improved, the prognosis for ovarian cancer is still not good. Within five years, patients have a significant chance of experiencing a return of the disease, and chemotherapeutic approaches are usually ineffective against recurrent ovarian cancer[32].

The endoplasmic reticulum is an essential cell organelle that is involved in protein folding, production, and modification of a variety of soluble and insoluble proteins[33]. Evolutionarily conserved cell stress response unfolded protein response is triggered by ER stress and sets off two downstream signaling cascades. Two mechanisms are involved in the correct folding of unfolded proteins: the first involves inducing genes expressing ER resident chaperones, and the second involves suppressing the commencement of protein synthesis. Three transmembrane ER stress sensors the basic leucine-zipper activating transcription factor ATF6, the kinase endoribonuclease IRE1α, and RNA-activated protein kinase-like ER kinase (PERK) carry out these operations[34]. The ER chaperone GRP78/BiP binds these stress sensors, keeping them dormant[9,10].

Cancerous cells do not stop proliferating because they are not well vascularized. They exhibit a high metabolism required to promote the growth of cancer and form a particular microenvironmental niche with low pH, hypoxia, and limited nutrition availability[35]. Cancer cells exhibit increased levels of PERK and its primary downstream effectors, which include nuclear factor-like 2 (Nrf2), ATF4, CHOP, and phosphorylated eukaryotic translation initiation factor 2α (p-eIF2α)[36,37].

In order to find new predictive biomarkers for epithelial ovarian cancer (EOC), Samanta et al. identified the expression of ER stress-associated proteins (GRP78, ATF6, and PERK), which they then connected with the clinical prognosis of EOC. They used immunohistochemistry to measure the expression levels of ER-associated proteins. It has been noted that specimens of EOC had considerably higher expression levels of GRP78, ATF6, and PERK in comparison to normal tissues[38].

SVIP has a highly conserved VIM as well[39]. Using p97/VCP as bait, a yeast two-hybrid screen was used to isolate SVIP. Cells become vacuolized when SVIP is overexpressed, however, its physiological significance is unclear [40]. On the cytosolic surface of the ER membrane is where SVIP is located. By interacting with p97/VCP, which is essential to the ERAD pathway and is known to function independently of ubiquitin, it suppresses this route. Previous studies have shown that SVIP blocked p97/VCP from using proteasomal degradation to remove misfolded proteins from the ER. Prolonged ER stress, a condition marked by the accumulation of misfolded proteins in the ER, significantly increases SVIP, which is expected to severely impede ERAD[28].

SVIP was discovered as a novel adapter protein for p97/VCP. It has been noted that when SVIP was overexpressed in cells, resulted in significant vacuolation and deformation of the ER and microtubules[41]. A tumor inhibitory activity of SVIP was discovered in a recent study and increased endoplasmic reticulum stress and the inhibition of cancer growth have been linked to its expression[42]. It was observed that androgen downregulates SVIP in glioma cells, and this finding suggests SVIP is a novel target for p53wt gliomas[43].

According to recent research, SVIP might play a role in the development of cancer. First, in prostate cancer cells treated with androgen, SVIP levels were downregulated, but other ERAD machinery components were elevated which was revealed to be positively associated with prostate carcinogenesis. Moreover, SVIP expression in various prostate cell lines was compared, and it was found that SVIP is highly expressed in androgen-dependent prostate

cancer cells (LNCAP, 22RV1) but not in androgen-independent cell lines (PC3, DU145) or non-tumorigenic prostate cell lines, such as BPH1 and RWPE1 normal prostate epithelial cell lines[44].

Recently, studies on cancer biology have begun to focus on SVIP. It has been documented that in certain cancer cells, particularly head and neck cancer, SVIP experiences DNA hypermethylation-associated silencing. SVIP demonstrated the characteristics of a tumor suppressor and its suppression in cancer cells with hypermethylated SVIP. Interestingly, the SVIP promoter CpG island was most frequently found to be unmethylated in the other cancer types, including prostate and breast cancers, except head and neck (50%), esophageal (23%), cervical (21%) and hematological malignancies (14%), particularly B cell lymphoma (33%)[42].

In the current study, we investigated the existence and cellular location of SVIP and GRP78, ATF4, PERK in OVCAR-3 cells. SVIP and GRP78, PERK, ATF4 cytoplasmic expressions were found in OVCAR-3 cells using the immunofluorescence technique. Furthermore, we employed double immunostaining to ascertain the overlaps between SVIP and GRP78, PERK, ATF4. The colocalization of SVIP with GRP78, PERK, and ATF4 in the cytoplasm of the OVCAR-3 cells was demonstrated by the immunofluorescence data.

In conclusion, although we showed the cellular localization of the SVIP and ER stress markers in ovarian cancer cell lines in the current study, more studies are needed to determine the connection between the SVIP and the UPR sensor proteins.

Ethics Committee Approval Information:
Since a cell line was used in our study, there is no need for ethics committee approval.

Researcher Contribution Statement:
Idea and design: E.A.; B.K.; Data collection and processing: E.A.; Analysis and interpretation of data: E.A.; Writing of significant parts of the article: E.A.; B.K.
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