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Activation of the MEK – S6 pathway in high-grade ovarian cancers

Aviva P. Ventura, BS[1,*], **Sabarinath Radhakrishnan, MD**[1,*], **Ann Green, BS**[1], **Sunitha K. Rajaram, PhD**[1], **April N. Allen, BS**[1], **Kathy O'Briant, MS**[1], **Michèl Schummer, PhD**[1], **Beth Karlan, MD**[2], **Nicole Urban, ScD**[1], **Muneesh Tewari, MD, PhD**[3,4], **Charles Drescher, MD**[1], and **Beatrice S. Knudsen, MD, PhD**[1,5]

[1] Division of Public Health Sciences, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA 98109, USA

[2] Women's Cancer Research Institute at Cedars-Sinai Samuel Oschin Comprehensive Cancer Institute, Los Angeles CA 90048

[3] Division of Human Biology, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA 98109, USA

[4] Division of Clinical Research, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA 98109, USA

[5] Department of Pathology, University of Washington, Seattle, WA 98105, USA

Abstract

The primary objective of this study is to demonstrate the activation and analyze the regulation of the MEK- S6 kinase pathway in high-grade ovarian cancer. Phospho-ERK (pERK), a direct substrate of MEK and two phosphorylation sites on the ribosomal protein, S6, Ser235/236 and Ser240/244, which are both targeted by the MEK and PI3-kinase/AKT pathways, were analyzed in 13 cell lines, 28 primary cancers and 8 cases of cancer cells from ascites. In primary cancers, ERK and S6 phosphorylation was measured by immunohistochemistry (IHC). pERK, pS6, pAKT and p4EBP1 were also measured by Western blotting (WB). The regulation of S6 phosphorylation by the MEK and PI3-kinase pathways was determined in ovarian cancer cell lines. We observed frequent pERK expression in primary ovarian cancers (100 % by WB, 75% by IHC) but not in ovarian cancer cells from ascites (25% of cases by WB). The activation of the AKT pathway, measured by pAKT expression occurred in 7 cases of primary ovarian cancer by WB, but in none of the ascites samples. In ovarian cancer cell lines, the MEK pathway had a greater effect on S6 phosphorylation in cells without hyperactive AKT signaling. Our data suggest that MEK is a potential drug target in high-grade ovarian cancer, however cancer cells with hyperactive AKT and cancer cells in ascites may be less responsive to MEK inhibition. The phosphorylation of S6 as a specific biomarker for either MEK or PI3-kinase pathway activation should be used with caution.

Corresponding author: Beatrice Knudsen, MD, PhD, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Mailstop M5-A864, Seattle, WA 98109, Tel: (206) 667-1823, Fax: (206) 667-2537, bknudsen@fhcrc.org.
[*]These authors contributed equally to the study

Conflict of Interest Statement
The authors declare that there are no conflicts of interest.

Keywords

ovarian cancer; pathway activation; MEK; AKT; S6

Introduction

Ovarian cancer is one of the most lethal malignant diseases in women, because it is often diagnosed at a late stage. Most patients have evidence of metastatic spread beyond the ovaries (FIGO stages III and IV disease) from cancers with high-grade histologies, of which 80% are of the papillary serous type [1]. While many cancers respond well initially to the standard therapy with platinum and taxane agents, durable responses are uncommon [2]. There is a large need to test new treatment strategies to eradicate ovarian cancer cells and prevent cancer recurrence [3]. A broad range of drugs that target specific pathways have become available. However, the activation of these pathways in ovarian cancers from patients and their relevance for promoting ovarian cancer growth are incompletely understood. To treat patients more effectively, companion biomarkers are needed for treatment decisions and to follow the response to treatment.

One pathway that is often aberrantly activated in high-grade ovarian cancers is the PI3-kinase pathway [4]. Pathway activation is observed in approximately 50% of cancers, either through mutations or aberrant expression of PI3KCA, AKT2, or PTEN [5–7,8]. While PI3KCA mutations are frequent in clear cell carcinoma[9], in serous ovarian cancers, genomic amplifications of PI3KCA and AKT2 are more common [10]. In contrast to the PI3-kinase pathway, the RAS – RAF – MEK pathway has received little attention in high-grade ovarian cancer because mutations are rare [11–13]. Despite the lack of mutations, MEK is frequently activated in high-grade serous ovarian cancers [14,15]. MEK inhibitors were effective in cancers in which MEK activation occurred in the absence of RAS or RAF mutations [16] and MEK inhibitors have been noted for their low toxicity [17]. Altogether, these data stimulated a new wave of interest in MEK as a therapeutic target [19,20].

MEK phosphorylates the extracellular signal-regulated kinase (ERK) [18,21] and ERK phosphorylation (pERK) is commonly used as a surrogate marker for MEK activity. In addition, ERK regulates protein synthesis through the p70 and p90 ribosomal S6 kinases (RSK, S6K1/2) [22, 23]. RSK and S6K1/2 phosphorylate S6 at two C-terminal clusters, Ser235/236 [24] and Ser240/244 [25]. These phosphorylation sites are commonly used as a surrogate measure for mTOR and S6-kinase activity because neither of these kinases can be accurately measured by immunohistochemistry (IHC) in patient tissues. While phosphorylation at the two S6 sites is used interchangeably in IHC studies, it is unclear whether the sites are phosphorylated in a concordant manner and whether phosphorylation occurs through the same upstream pathways.

In this study, we examine the expression of ERK, AKT, and S6 phosphorylation in ovarian cancer cell lines, tissues from high-grade ovarian cancers, and cancer cells from ascites. We observe frequent expression of pERK (100%) in primary ovarian cancers by Western blotting (WB), but less frequent expression (75%) by IHC. In ovarian cancer cell lines without a hyperactive AKT pathway, the phosphorylation of S6 on Ser235/236 was regulated by the ERK/mTOR pathway, while phosphorylation on Ser240/244 was primarily regulated by the PI3-kinase/mTOR pathway. Surprisingly, the expression of pERK in cancer cells isolated from ascites only occured in 25% of cases and none of the cases expressed pAKT. Analysis of MEK-S6 and AKT-S6 pathways in ascites cells revealed a more limited amount of phosphoprotein expression in ascites cells compared to the primary cancers, with the caveat that cancer cells from ascites and primary tumors were not from the same

patients. The data suggest that activation of the MEK and AKT pathways are attenuated in ascites cells and as a result, drugs that effectively inhibit active pathways in primary cancers may be ineffective against cancer cells in ascites.

METHODS

Cell lines, antibodies and chemicals

The following cell lines were purchased from ATCC: ES-2, CaOV3, SKOV3, OV-90, TOV-21G, and TOV-112D. A2780, A1847, IGROV1, OVCAR3, OVCAR5, OVCAR10, and PEO-1 were obtained from the Pacific Ovarian Cancer Research Consortium (POCRC). Dr. George Coukos at the Abramson Family Cancer Research Institute at the University of Pennsylvania supplied the 2008 cell line. The mutation status of ES-2, IGROV1, OVCAR3, SKOV3, and TOV-21G has been analyzed[26–28]. Cells were cultured using the media formulations that are recommended by ATCC. The following antibodies were purchased from Cell Signaling Technologies (Danvers, MA): pS6 ribosomal protein (Ser 235/236) - clone 91B2 rabbit monoclonal antibody; pS6 (Ser240/244) - rabbit polyclonal antibody; pp44/42 ERK (Thr202/Tyr204) - rabbit polyclonal antibody; pAKT(Ser473) – rabbit polyclonal antibody; p4EBP1 (Thr37/46) rabbit polyclonal antibody. Cytokeratin 18 rabbit polyclonal antibody was purchased from Abcam (Cambridge, MA) and CK8 mouse monoclonal antibody [35βH11] was purchased from DAKO (Glostrup, Denmark).

All protein lysates for Western blot analysis were prepared in a cell lysis buffer (RIPA) comprised of 0.5% Deoxycholic acid, 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1% Triton, and 0.1% SDS. Roche mini protease inhibitor cocktail (Indianapolis, IN)and phosphatase inhibitors (Calbiochem, EMD, Darmstadt, Germany) were added according to manufacturer's instructions.

LY294002 was purchased from Promega (Madison, WI); rapamycin and U0126 were obtained from Cell Signaling Technologies. Stock solutions were prepared in diluents according to the manufacturer's recommendations.

Tissue collection and processing

Frozen tissues and corresponding formalin-fixed paraffin embedded (FFPE) tissue blocks from human ovarian tissues were provided by the POCRC. Under an IRB-approved protocol from the Fred Hutchinson Cancer Research Center, patients were consented to donate their tissues. POCRC staff working at the Marsha Rivkin Center for Ovarian Cancer Research collected tissue and ascites samples from patients, who received their treatment at the Swedish Medical Center in Seattle, WA. The diagnosis of ovarian cancer was confirmed by a board certified Gyn-pathologist at the Swedish Medical Center through centralized review of slides from formalin-fixed and paraffin-treated sections and the information about the histologic subtype of ovarian cancers was obtained from the pathology report. Only cases with > 80% cancer were selected for the study. Adjacent FFPE tissue and frozen tissues were used for analysis. Tissues were typically processed and frozen within 30 minutes of excision from the patient. Snap frozen tissues were stored in liquid nitrogen until use. FFPE blocks were stored at room temperature.

Quality control of tissues for phosphorylation studies

Because protein phosphorylation is labile and de-phosphorylation is unpredictable, special precautions were taken to expedite the procurement of specimens for research (Supplementary Figure 1 Supplemental digital content http://links.lww.com/AIMM/A4). Tissues were cut to the size of a pea to decrease the time of infiltration with formalin. In addition, the same instrument was used for all FFPE tissue processing and the program

settings of the tissue processor were consistent. Frozen tissue samples underwent a quality control (QC) evaluation for integrity of protein and phosphorylation before proceeding with the data analysis of WB results. A sample passed QC if bands of beta-actin and S6 were present, demonstrating the absence of significant protein degradation in the sample. In this study, 6/24 samples were excluded from further analysis because of lack of beta-actin or S6 bands.

Isolation of cancer cells from ascites and determination of purity

Ascites fluid containing cancer cells was provided by the POCRC. The ascites was from a different group of patients than the primary tumor samples. Ascites was collected into a container that contained heparin and was processed either on the same day or after storage at 4°C overnight. Raw ascites fluid was centrifuged at 2,000 rpm for 5 minutes to pellet cells. The supernatant was removed and cells were then treated twice with RBC Lysis solution (156 mM sodium chloride, 10 mM sodium bicarbonate, 0.1 mM disodium EDTA) for 20 minutes each with gentle rocking. Each treatment was followed by centrifugation and removal of supernatant. Cells were washed with cold PBS, collected by centrifugation and loaded onto a Ficoll gradient (GE Healthcare, Piscataway, NJ) to remove dead cells, and/or Percoll Gradient (GE Healthcare, Piscataway, NJ) (30%, 40%, 50%, 60%) to remove inflammatory cells. The enrichment and viability of cancer cells were monitored during the procedure. The purity of cancer cells was evaluated by cytokeratin 8 (CK8) staining in a cytospin preparation.

Immunohistochemistry

IHC was performed using a standard procedure. Briefly, antigen retrieval in DAKO Target Retrieval Solution occurred for 20 minutes in a Black and Decker vegetable steamer. After blocking for 10 minutes in DAKO serum-free block, antibodies were used at a 1:100 dilution. Slides were developed using the Vectastain ABC kit (Vector Labs, Burlingame, CA) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a substrate.

The quality of immunohistochemical staining was evaluated using endothelial cells as an internal positive control. The expression of pERK and pS6 was quantified on a scale of 0 – 300 as previously described [29]. The intensity was recorded on a scale from 0 to 3 and multiplied by the percentage of cells at a given intensity.

Treatment of cells with pathway inhibitors

Cells were grown to 80% confluence in medium with 10% fetal calf serum (FCS). For treatment with U0126 MEK inhibitor, cells were transferred to 0.5% FCS for 8 hours and then treated with 10 micromolar U0126 for 16 hours. Treatment with the PI3K/lipid kinase inhibitor LY294002 and mTOR inhibitor rapamycin was carried out in 10% FCS to avoid drug toxicity (data not shown), except for TOV-21G, which were treated in 0.5% FCS. The rapamycin concentration was 10 nanomolar, the LY294002 concentration was 10 micromolar, except for SKOV3 cells, which were treated with 20 micromolar. Control plates were treated with an equal amount of diluent. Treatments using LY294002 and rapamycin were conducted for 16 hours

Western Blotting

Frozen tissues were lysed in 400μl RIPA buffer containing Roche mini protease inhibitor cocktail and phosphatase inhibitor cocktail Set II using a Qiagen TissueLyser (Valencia, CA) set at 30Hz for 3 minutes. Total tissue or cell extracts were rocked on a nutator at 4°C for 10 minutes and then centrifuged at 13,000 rpm at 4°C for 10 minutes. Protein from the

supernatant was measured using a Bradford assay (Bio-Rad, Hercules, Ca). An equal amount of RIPA buffer was loaded into the blank and standards.

Fifty micrograms of protein were separated by gel electrophoresis and Western blotting was performed as described [30]. After blocking the membrane in 5% milk, all primary antibodies were used at 1:1000 in 5% BSA at 4oC overnight with gentle rocking except for pAkt (Ser 473), which was diluted 1:500. Horseradish peroxidase conjugated secondary antibodies (Jackson Immunolabs, West Grove, PA) were used at 1:10,000 in milk for one hour at room temperature. The Western blot bands were visualized by chemiluminescence. Each sample was analyzed at least twice. All Western blots were reprobed with a beta-actin antibody.

Measurement of signal intensity of WB bands

The Image Quant TL (Amersham Pharmacia Biotech) software was used for quantification. Bands were marked using the band detection tool. A region of interest around the largest band was drawn as a measurement box. This box was applied to all lanes. Background was subtracted using the "*rolling ball*" tool and intensities were recorded.

Statistical analysis

Statistical calculations were performed using Microsoft Excel. Correlations were calculated using the Pearson's correlation coefficient.

Results

Expression of pERK and pS6 in tissues of high-grade ovarian cancer from patients

To determine the activation of the MEK – S6 pathway in high-grade ovarian cancers, we measured the phosphorylation of ERK on Thr202/Tyr204 as a surrogate for MEK activity, and the phosphorylation of S6 on Ser235/236. Because we worried about the quality of formalin fixed tissues, we first compared phosphoprotein expression in adjacent formalin-fixed (FFPE) and snap-frozen tissues. pERK and pS6 were measured by IHC in 28 FFPE tissues and by WB in 18 frozen tissues.

The cases for the WB analysis consisted of ovarian cancers with the following histologies: 6 endometrioid, 2 clear cell and 10 high-grade serous. To Figure 1A shows that all 18 cases express phosphorylated ERK proteins and that 12 cases express high pS6. An additional 3 cases demonstrate little S6.

IHC-scores of 28 high-grade ovarian cancers are shown in Supplementary Table 1 supplemental digital content http://links.lww.com/AIMM/A6. Scores were generated on a continuous scale between 1 and 300, multiplying the intensity and percentage of positive cancer cells [29]. Similar to a previous IHC study [14], we used endothelial cells as an internal positive control (Figure 1D, panels a and b). A positive IHC score (> 30) for pERK was obtained in 21/28 cases (75%). The IHC score was high (> 200) in 6 cases, medium (100 – 200) in 10 cases and low (30< IHC score < 100) in 5 cases. There was no association between histologic subtype of the cancer and staining intensity. pERK expression was heterogeneous with cell-to-cell differences (Figure 1D, panel c); In 3 cases, pERK expression was mostly nuclear, whereas in the other cases it was both nuclear and cytoplasmic (Figure 1D, panel d); In half of the cases, expression was increased at the interface of the cancer with the stroma compared to the inside of the cancer. For the 18 cases with parallel IHC and WB data, intensities of the IHC and WB results are shown in Figure 1B. The correlation coefficient between pERK IHC and WB was $\sigma = 0.47$, only a modest concordance despite the good tissue quality.

For pS6 (Ser235/236) a positive IHC score (> 30) was observed in 20/27 cases. High expression (> 200) was found in 4 cases, medium (100 – 200) in 5 cases and low (30 < IHC stain < 100) in 11 cases (Supplementary Table 1 http://links.lww.com/AIMM/A6). The pS6 staining was independent of the histologic subtype of the cancer, increased at the interface between stroma and cancer in about one third of the cases (Figure 1D, panel e), and was heterogeneous with high cell-to-cell variability (Figure 1D, panel f). In Figure 1C, we compare pS6 IHC and WB results. The correlation coefficient for pS6 between IHC and WB analysis was $\sigma = 0.16$. The poor correlation may be caused by the biological variability of pS6 expression in adjacent tissue samples. The association of pERK and pS6 staining was determined in 27 cases and the correlation coefficient was $\sigma = 0.098$, thus there was no obvious correlation between MEK activity and pS6 expression.

Expression of pAKT, p4EBP1 and pS6 in primary ovarian cancers

pS6 and p4EBP1 are well established targets of AKT/mTOR pathway activation in cultured cells[31]. We questioned whether ovarian cancers from patients coexpress pAKT, pS6, and p4EBP1. Frozen lysates were analyzed by Western blotting and the data are shown in Supplementary Table 2 supplemental digital content http://links.lww.com/AIMM/A7. Consistent with results from another study[32], 7/18 (39%) of ovarian cancers in our cohort expressed pAKT. However, several pAKT negative cases expressed p4EBP1 or pS6. The greater frequency of p4EBP1 (11/18) and pS6 (16/18) expression compared to pAKT expression suggests that additional mechanisms may lead to an increased steady-state 4EBP1 and S6 phosphorylation [33,34].

Regulation of S6 phosphorylation by MEK and AKT pathways in ovarian cancer cell lines

S6 phosphorylation occurs through the ERK-RSK as well as through the PI3K/AKT/mTOR/RSK pathways [23, 25, 35,36]. Two phosphorylation clusters exist in the C-terminus of S6, Ser235/236 and Ser240/244 and are often used interchangeably for analysis in tissues to assess the translational activity of the mTOR pathway. To determine how the MEK and AKT pathways regulate the phosphorylation of S6 clusters, we screened 13 ovarian cancer cell lines and selected 8 that express pERK (Supplementary Figure 2 supplemental digital content http://links.lww.com/AIMM/A5). 4 have constitutive AKT activation (AKT ON): IGROV1, SKOV3, TOV21G, OVCAR10, while the other 4 did not (AKT OFF): A1847, OVCAR5, PEO-1, TOV112D. To analyze the MEK-S6 pathway, cells were treated with the MEK1/2 inhibitor, U0126 and to analyze the PI3K/mTOR pathway, cells were treated with LY294002 and rapamycin. All drug treatments were carried out for 16 hours to simulate chronic exposure to the drug. The drug concentrations were adjusted to inhibit ERK and S6 phosphorylation. At their effective doses, the drugs did not cause a significant amount of cell detachment (toxicity) during the treatment period (data not shown).

First we evaluated the response of the cell lines to inhibition of MEK (Figure 2A). Upon treatment with U0126, pERK expression was abolished in all cell lines and both S6 phosphorylation sites were affected. The Ser235/236 site was more responsive to than the Ser240/244 site. Next we tested the effects of LY294002 and rapamycin (Figure 2B). Treatment with LY924002 decreased pAKT expression in the AKT ON cell lines (data not shown). In addition, phosphorylation of pS6 (Ser235/236) and pS6 (Ser240/244) were inhibited by LY294002 as well as by rapamycin. From these results, we conclude that both MEK - mTOR and PI3K - mTOR pathways are required for S6 phosphorylation in AKT OFF cells, but that MEK is dispensable in AKT ON cells (Figure 3). Thus, the regulation of phosphorylation differs between AKT ON and AKT OFF cells.

Expression of pERK and pS6 in ovarian cancer cells isolated from ascites

Because, in most patients with ovarian cancer, at the time of diagnosis and treatment cancer cells have disseminated into the peritoneal cavity, the analysis of ovarian cancer cells in ascites is an important factor in the assessment of the treatment response to a new drug. In particular, the activation of the MEK and AKT pathways may differ in cancer cells in ascites compared to primary tumors because the pathways require cell adhesion to the extracellular matrix as a co-stimulus for protein phosphorylation[37]. We obtained ascites fluid and isolated the cancer cells to measure pathway activation. The ascites fluid was from a different group of patients than the primary tumors. In 8 cases of serous cancer, a sufficient amount of protein was isolated from the purified ascites cells to perform Western blot analysis.

To determine the amount of cancer cells in the sample, we measured cytokeratin 18 (CK18), which is expressed in ovarian cancer cells, but not in inflammatory cells. CK18 was expressed in 7/8 cases (Figure 4). Further, all samples expressed pS6 (Ser240/244), suggesting that phosphorylation was maintained during the sample preparation. Pathway activation was variable across patients. In contrast to the ubiquitous expression of pERK (10/10) in the primary serous ovarian cancers only 2/8 cases expressed pERK in ascites derived tumor cells. While 6/10 (60%) serous primary cancers expressed pS6 (Ser235/236) only 3/8 (37%) ascites cancer cells were positive. While AKT phosphorylation was observed in 2/10 primary serous cancers, none of the ascites cases expressed pAKT. In addition only 1/6 cases from ascites expressed p4EBP1 compared to 8/10 primary serous cancers (data not shown). Thus, compared to the primary cancers, pathway activation in cancer cells in ascites was markedly diminished.

Discussion

High-grade ovarian cancers pose a particular challenge to treatment with targeted agents, because of the heterogeneous nature of this cancer type. Inhibitors of the MEK kinase, a kinase in the RAS – ERK pathway have recently been developed and found to have low toxicity profiles [16,38–42]. MEK inhibitors could be considered for the treatment of high-grade ovarian cancers that possess an active MEK kinase. To determine the frequency of MEK activation, we measured phosphorylation of ERK as a surrogate marker. We measured pERK in 28 high-grade ovarian cancers by IHC and in a subset of 18 cancers by WB. Tissues underwent a stringent quality control to assure the validity of the measurements. pERK was expressed in all high-grade primary ovarian cancers, including 10 cancers with serous histology, but only in 2/8 cases of cancer cells in ascites. It is unlikely that the absence of protein phosphorylation in ascites cells was caused by the long processing period of ascites samples, since the S6 (Ser240/244) phosphorylation site was expressed in all cases and pERK and pS6 negative cases express other phosphorylated proteins, such as pAMPK or pp38 (data not shown). It is more likely that the restricted activation of signaling pathways is due to decreased of clonal diversity of ascites cells, or a lack of stimulation by the stroma. This hypothesis is supported by the fact that we observed more pathway activation in cancer cells at the interface between the cancer and the stroma compared to the inside of the cancer in primary tumors (Figure 1D), (Supplementary Table 1 http://links.lww.com/AIMM/A6).

The primary objective of this study was to evaluate whether S6 phosphorylation provides a marker of activation of the MEK-ERK pathway. First we determined whether both S6 phosphorylation clusters are regulated by MEK and observed that MEK can regulate phosphorylation at both sites in cancer cells that do not possess a hyperactive AKT pathway. Based on these results we performed a subanalysis of the pAKT negative primary cancers and found no correlation between pERK and pS6 ($\sigma = 0.124$). It is likely that the absence of a correlation is due to the considerable regional biological variability of S6 phosphorylation

in ovarian cancers. This suggests that the microenvironment plays an important role. Altogether the data demonstrate that pS6 should cautiously be utilized as a marker of MEK or AKT pathway activation.

The largest immunohistochemical study of pERK expression in 207 ovarian cancers found a lower frequency of expression in high-grade compared to low-grade cancers [43]. In that study, expression was observed in all low-grade tumors with KRAS and BRAF mutations. In addition, 41% of high-grade serous ovarian cancers expressed pERK by immunohistochemical analysis. While our study had a smaller sample size, we observed higher frequency of pERK expression by IHC (75%) in high grade cancers of mixed histologies. The difference may be related to the stringent QC of tissues in our study. Altogether the high prevalence of MEK activity in our study increases the promise of success of MEK inhibitors in high grade ovarian cancer, in particular because of the recent development of novel MEK inhibitors with affinities in the nanomolar range [20,44,45].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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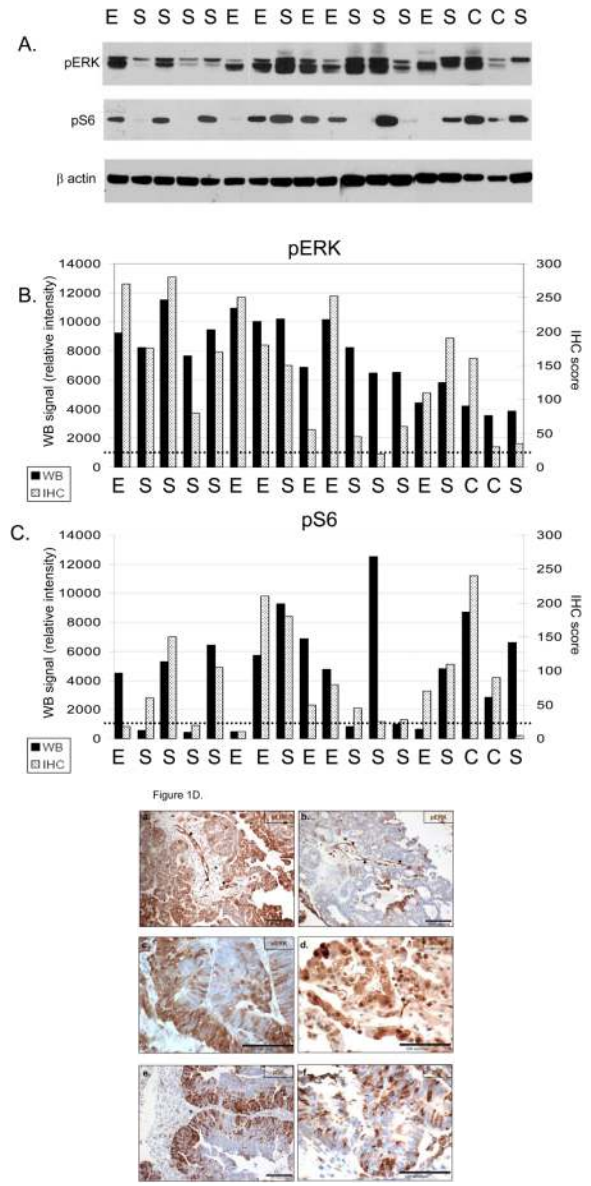

Figure 1. Phosphorylation of ERK and S6 in primary ovarian cancers

A. ***Western blot analysis.*** Fifty micrograms of whole cell lysate from frozen tissue samples of serous ovarian cancers (S), endometrioid ovarian cancer (E) or clear cell ovarian cancer (C) were analyzed by Western blotting. ERK phosphorylation was measured on Thr202/ Tyr204 and S6 phosphorylation on Ser235/236. The specificity of the pERK antibody is demonstrated in Figure 2A and the specificity of the pS6 antibody in Figure 2B. Samples were analyzed 2 – 3 times on different Western blots and a representative image is shown in the figure.

B. ***Comparison of pERK expression by Western blotting and immunohistochemistry.*** The Western blot signals (solid black bars) were quantified by image analysis as described in Material and Methods. The IHC scores in adjacent tissues (dotted bars) were obtained by multiplying intensity (on a scale of 0 – 3) and percentage of cells stained. The horizontal dotted line indicates the threshold of detection.

C. ***Comparison of pS6 expression by Western blotting and immunohistochemistry.*** The Western blot signals (solid bars) were quantified by image analysis. The IHC scores (dotted bars) are from adjacent tissues. The horizontal dotted line indicates the threshold of detection.

D. ***Examples of pERK and pS6 IHC in ovarian cancer.*** *a. High expression of pERK.* pERK expression in cancer cells is visualized by the brown color. The arrow heads point to vessels and the asterix denotes the stroma. *b. Low expression of pERK.* Only a few cancer cells stain brown. Arrow heads point to vessels as the internal positive control. *c. Cytoplasmic expression of pERK. d. nuclear expression of pERK. E. Expression of pS6 at the stroma-cancer interface.* The asterix marks the stroma *f. Heterogeneous expression of pS6 in cancer cells.*

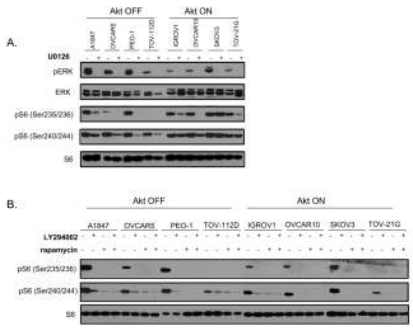

Figure 2. Regulation of S6 phosphorylation in ovarian cancer cell lines

A. ***Regulation of S6 phosphorylation by MEK.*** Ovarian cancer cells lines with AKT ON or AKT OFF were treated with the MEK inhibitor U0126 for 16 hours to mimic chronic treatment conditions. The inhibition of MEK was monitored by measuring pERK. Samples were then analyzed for pS6 (Ser235/236) and pS6 (Ser240/244) expression. Western blots were also probed for total ERK and S6 proteins to verify that equal amounts of sample were loaded. The Western blot was repeated twice with the same result. The figure shows a composite.

B. ***Regulation of S6 phosphorylation by PI3-kinase and mTOR.*** Cells lines with AKT ON or OFF were treated with LY294002 and/or rapamycin for 16 hours to mimic chronic treatment conditions. Samples were probed for expression of pS6 (Ser235/236) and pS6 (Ser240/244). Western blots were probed for total S6 proteins to verify equal amounts of protein loading in the lanes. The Western blot was repeated once with the same result and the image consists of a composite.

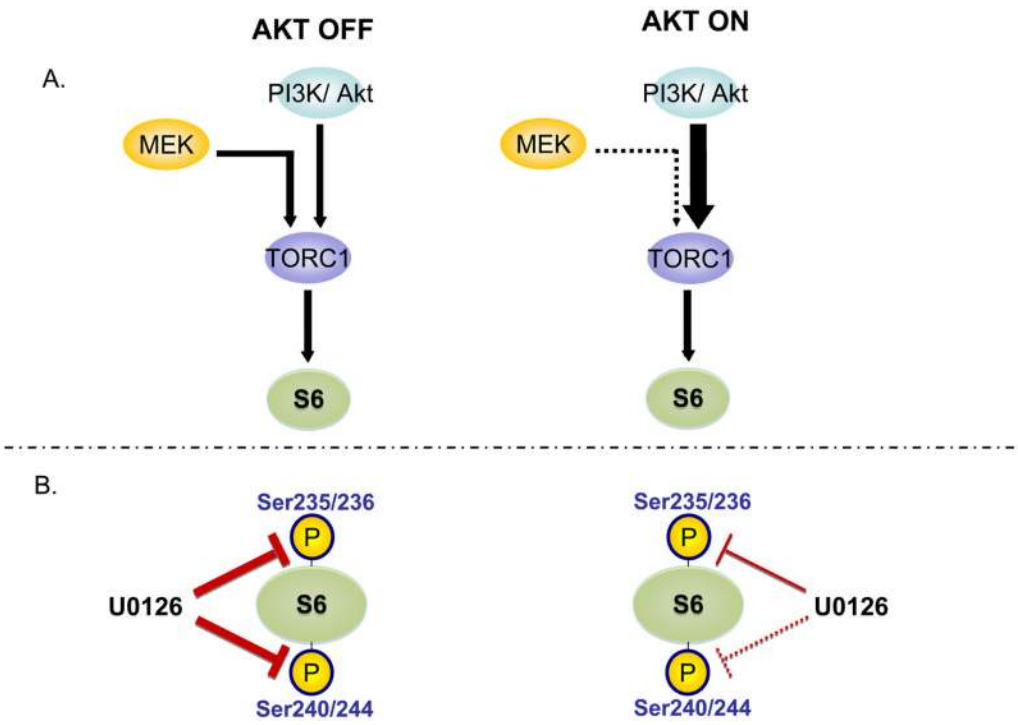

Figure 3. Schematic of pathways responsible for S6 phosphorylation in ovarian cancer cell lines

A. ***Pathways in cell lines with and without constitutive pAKT expression.*** The left column shows pathway activation in AKT OFF cell lines and the right column in AKT ON cell lines. The phosphorylation of S6 was equally strong in AKT ON and AKTOFF cell lines. In the AKT OFF cell lines, both MEK and PI3K/mTOR mediate pS6 phosphorylation. In the AKT ON cell lines, the AKT pathway is dominant and MEK pathway has a lesser effect.

B. ***Regulation of two phosphorylation sites on S6 by MEK.*** Inhibition of MEK affected both phosphorylation sites in AKT OFF cell lines (thick red bars). In AKT ON cell lines, U0126 was more effective at the Ser235/236 site than the Ser240/244 site (thin and dotted red bars, respectively), but overall U0126 was less effective in AKT ON compared to AKT OFF cell lines.

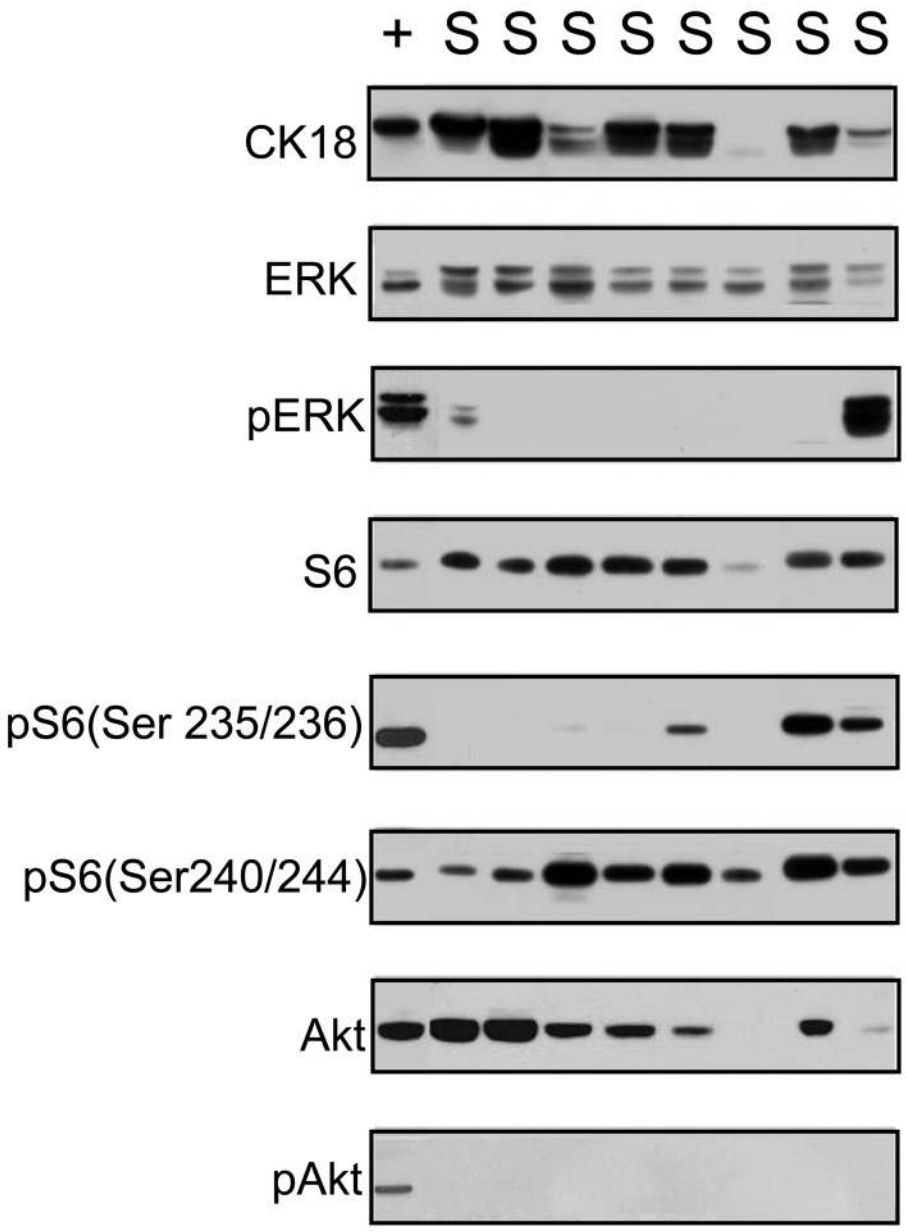

Figure 4. Expression of pERK and pS6 in ovarian cancer cells from ascites

Fifty microliters of whole cell lysate from ovarian cancer cells derived from patient ascites were loaded in each lane. All patients had primary serous ovarian cancer (S). The amount of cancer cells was determined with the CK18 antibody. Samples were analyzed for pERK, pS6 (Ser235/236), pS6 (Ser240/244) and pAKT expression. S6 was used as a loading control. The WB analysis was repeated twice for all samples and three times for some. A representative result is shown.