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# Differential Expression of mTOR Signalling Components in Drug Resistance in Ovarian Cancer

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**Abstract.** *Background/Aim: A limitation to successful cancer chemotherapy treatments is the acquisition of drug resistance. In advanced-stage ovarian cancer, the mammalian target of rapamycin (mTOR) pathway is up-regulated, and inhibition of this pathway increases chemosensitivity in ovarian carcinoma cell lines. In this study, the expression of DEPTOR, mTOR, RICTOR, RAPTOR and S6 kinases were investigated in SKOV-3 and PEO1 parental and the paclitaxel-resistant (TaxR) SKOV-3TaxR and PEO1TaxR cell lines. Materials and Methods: RT-PCR, immunofluorescent analysis and Western blotting were carried out. Results: Quantitative RT-PCR revealed significant up-regulation of DEPTOR in both paclitaxel-resistant cell lines. SKOV-3TaxR exhibited down-regulation of RICTOR, RAPTOR and mTOR, whereas PEO1-TaxR showed down-regulation of RAPTOR and up-regulation of RICTOR and mTOR. Semi-quantitative RT-PCR analysis revealed marked changes in the expression of p70S6K splice variants mRNA in PEO1TaxR. Moreover, the phosphorylation status of p70S6K at Ser371 appears to be cell-type specific. Conclusion: We hypothesize that mTOR signalling may play a role in mediating paclitaxel resistance in ovarian cancer.*

Ovarian carcinoma is the gynaecological malignancy associated with the highest mortality in industrialised countries, with a reported 5-year survival rate of <30% (1). The prognosis for patients with ovarian cancer is determined by conventional factors such as surgical stage and histological grade and type. Nevertheless, to the best of our knowledge, no single molecular profile has helped identify the most aggressive tumours, nor aided in the guidance of suitable therapeutic strategies for specific patients. Many carcinomas activate growth factor receptor signalling pathways that exhibit genetic alterations involving either the receptor, or other factors that drive the proliferation (2). Interestingly, several pathways converge on the highly conserved serine/threonine kinase mammalian target of rapamycin (mTOR), which plays a central role in controlling cell growth (3).

Current treatments for newly diagnosed ovarian tumours centre on the use of platinum containing drugs such as cisplatin or carboplatin and often in combination therapy with paclitaxel (Taxol™). The treatment options for recurrent or advanced disease depend on whether tumours are resistant or refractory to previously used platinum-based drugs (4). Most patients with advanced disease will be treated with paclitaxel, or in combination with other agents, such as topotecan (5). One of the most important factors affecting patient survival is the development of drug resistance (6). Chemoresistance to agents such as paclitaxel can be acquired through a variety of mechanisms including altered drug metabolism, reduction in sensitivity to cell death stimuli (7), and alterations in microtubule dynamics (8, 9). The multidrug-resistant phenotype (MDR) mediated by ATP-binding cassette (ABC) transporters has been shown to be an important correlate in taxane resistance in cultured cell line models. ABCB1 (P-glycoprotein) is the drug transporter most frequently associated with paclitaxel- and docetaxel-resistant cell lines. To date, the clinical relevance of this in a number of tumour types, including breast and ovarian, has not been adequately verified (10).

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The PI3K-Akt-mTOR pathway is activated in advanced-stage disease (2, 11) and inhibition of this pathway with inhibitors to Akt or its downstream effector, mTOR, increases chemosensitivity to paclitaxel in ovarian carcinoma cell lines (12, 13). Cell signalling by mTOR plays a critical role in protein synthesis and proliferation of both normal and malignant cells (14, 15). mTOR and one of its substrates, S6 kinase, have been shown to be activated in ovarian cancer cell lines (16, 17). Inhibition of the mTOR pathways has antiproliferative effects. Rapamycin inhibits the growth of a broad spectrum of malignancies including pancreatic cancer, leukaemia and B-cell lymphoma, (18). Inhibitors of mTOR are known to increase chemosensitivity in a variety of tumours including ovarian cancer cell lines (12, 19, 20). Previous studies indicate that rapamycin may act as a substrate for the MDR transporter P-glycoprotein and consequently limit its utility in some tumours (21). Treatment of several ovarian cancer cell lines with the rapamycin analogue RAD001 (an inhibitor of mTOR) resulted in dose-dependent growth inhibition (22). Furthermore, in a transgenic mouse model of ovarian cancer, RAD001-treated mice exhibited a delay in cancer progression (11). Recently, a novel regulator of mTOR signalling has been described, named DEPTOR (DEPDC6). This molecule interacts with mTOR *via* its PDZ domain. In a series of elegant experiments, Peterson *et al*., showed that DEPTOR interacts with both mTORC1 and mTORC2 complexes. Inhibition of mTORC1 and overexpression of DEPTOR relieves mTORC1-mediated inhibition of PI3K. This leads to activation of PI3K and surprisingly activation of mTORC2-dependent outputs (23).

In this study, the expression and activity of mTOR signalling components in the context of paclitaxel-resistant ovarian cancer was investigated. SKOV-3 and PEO1 (parental) paclitaxel -sensitive and -resistant (TaxR) were used as representative models of taxane-refractory ovarian cancer.

## Materials and Methods

*Cell culture*. All cell culture reagents were obtained from Invitrogen (Paisley, UK) unless stated otherwise. PEO1 and SKOV-3 paclitaxel-sensitive and -resistant ovarian cancer cell lines, respectively, were developed by Dr Helen Coley, University of Surrey (10). For PEO1, RPMI-1640 10% foetal calf serum (FCS), 5% penicillin-streptomycin and 2 mM glutamine were used for cell culture at 37˚C with 5% $CO_2$. The SKOV-3 cell line was cultured in minimum essential medium/Earl's salts non-essential amino acid medium supplemented with 10% FCS, L-glutamine and the penicillin-streptomycin mixture at 37˚C with 5% $CO_2$.

*Chemosensitivity testing for the assessment of sensitivity to the mTOR inhibitor rapamycin*. Rapamycin was obtained from Sigma Aldrich (Poole, UK) and made up in sterile water as a stock solution. XR9576 (Tariquidar) was obtained from Xenova PLC (Slough, UK) and made up as a stock solution in dimethyl sulfoxide (DMSO) and stored as frozen aliquots. PEO1 cell lines were set up in 96-well plates at a cell density of $3\times10^4$ per ml and allowed to attach for 24 h. Rapamycin diluted in tissue culture medium was then added in increasing concentration in quadruplicate. Cells were then placed back in the incubator for 72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ( MTT) solution (5 mg/ml in phosphate buffered saline; PBS) was added to each well in 20 μl and left for 3-4 h to allow formazan crystal formation. Wells were aspirated and 200 μl of DMSO added to dissolve formazan crystals. The resulting purple coloration was measured at 540 nm in a plate reading spectrophotomer. Dose–response curves were constructed and used to determine the $IC_{50}$ dose (defined as the drug dose that gives rise to 50% loss of cell viability compared with untreated control wells). In limited experiments, we used the MDR modulator XR9576 at 100 nM in combination with rapamycin in order to ascertain any MDR reversal effects.

*RNA isolation, cDNA synthesis and PCR*. Total ribonucleic acid was isolated using an RNA extraction kit (Sigma Aldrich), according to the manufacturer's instructions. RNA concentration was determined by spectrophotometric analysis (NanoDrop; Thermo Scientific, UK) and agarose gel electrophoresis. RNA (500 ng) was reverse-transcribed into cDNA using 5 IU/μl RNase H reverse transcriptase (Invitrogen). PCR amplification was performed using *Taq* polymerase (Invitrogen) and oligonucleotide primers as previously described (24). A total of 28 cycles for each gene were performed, consisting of a denaturing step at 94˚C for 30 s, extension at 60˚C for 1 min and elongation at 72˚C for 1 min.

*Quantitative RT-PCR*. Relative expression of the genes of interest was assessed by quantitative PCR (Q-PCR) on an ABI Prism 7900HT Sequence detection system (Applied Biosystems) using SYBR® Green-PCR reaction mixture (Sigma Aldrich) and the primers as follows: DEPTOR (202 bp): forward: 5'-caccatgtg tgtgatgagca-3', reverse: 5'-tgaaggtgcgctcatacttg-3'; RICTOR (117 bp): forward: 5'-ggaagcctgttgatggtgat-3', reverse: 5'-ggcagcctgtttta tggtgt-3'; RAPTOR (170 bp): forward: 5'-actgatggagtccgaaatgc-3' reverse: 5'-tcatccgatccttcatcctc-3'; 18S RNA (155 bp): forward: 5'-aaacggctaccacatccaag-3', reverse: 5'-cctccaatggatcctcgtta-3'.

As a negative control, distilled water was used in place of the cDNA. RNAs were assayed from two to three independent biological replicates. The RNA levels were expressed as relative RQ values, using the parental cell line as calibrator. The Delta Delta Ct method was employed for comparing relative expression results between treatments in Q-PCR (25).

*Immunofluorescent analysis*. SKOV-3 and PEO1 cells were fixed in 4% paraformaldehyde for 10 min prior to washes in PBS and incubation with 10% bovine serum albumin (BSA) for 1 h. Cells were incubated for 1 h with an mTOR antibody (Santa Cruz Biotechnology, USA) at a 1:100 dilution in 1% BSA PBS. Cells were washed with PBS prior to an incubation with anti-rabbit IgG-fluorescein isothiocyanate (FITC)-conjugated antibody (Santa Cruz Biotechnology, USA) for 1 h. Slides were washed with PBS and mounted in Vectashield® Mounting Medium (Vector Labs) containing the dye 4,6-diamido-2-phenylindole (DAPI) to counterstain nuclei. Images were captured using a Plan Apo Neofluor X63 NA 1.25 oil objective (Zeiss) on a Zeiss Axiovert 200M microscope and viewed using AxioVision software, at set exposure times.

*Protein extraction from cultured cells*. SKOV-3 and PEO1 cells were cultured in 6-well dishes until 80% confluency. Cells were lysed with 300 μl 2× Laemmli Buffer (Sigma Aldrich) and denatured for 5 min at 100˚C before being cooled on ice.

*Western immunoblotting*. Samples were separated on an SDS-10% polyacrylamide gel and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked in TBS containing 0.1% Tween-20 and 5% dried milk powder (wt/vol), for 1 h at room temperature. After a brief wash with PBS-0.1% Tween-20, the nitrocellulose membranes were incubated with primary antibodies against phospho-mTOR (Ser2448), total mTOR, phospho-p70S6K (serine371/threonine389), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signalling, USA; Sigma Aldrich). The primary antisera were used at a 1:1000 dilution in PBS-0.1% Tween-20 overnight at 4˚C. The membranes were washed for 30 min with PBS-0.1% Tween-20, before incubation with the secondary anti-rabbit horseradish peroxidase-conjugated immunoglobulin (1:2000) for 1 h at room temperature and further washing for 30 min with PBS-0.1% Tween-20. Antibody complexes were visualised as previously described (26).

*Statistical analysis*. For the quantitative PCR, the following equations were used: $\Delta Ct=Ct_{(gene\ of\ interest)}-Ct_{(house\ keeping\ gene)}$, $\Delta\Delta Ct=\Delta Ct_{(sample)}-\Delta Ct_{(calibrator)}$, relative quantity $(RQ)=2^{-\Delta\Delta Ct}$. RQ value was set at 1 for the parental SKOV-3 and PEO1 cells when compared to paclitaxel-resistant (TaxR) ones. Data are reported as the mean±SD of three or four measurements. Statistical analysis was performed by Student's *t*-test and by ANOVA. $P<0.05$ was regarded as significant.

## Results

*Rapamycin sensitivity of PEO1 parental and paclitaxel-resistant PEO1TaxR cells*. The half maximal inhibitory concentration ($IC_{50}$) value for rapamycin in PEO1 was 25.3±3.9 μM and for PEO1TaxR was 22.8±3.4 μM, *p*-value=0.175 (n=4). The $IC_{50}$ value for the combination of rapamycin with XR9576 was unchanged for both cell lines, at 25.1 and 22.4 μM for PEO1 and PEO1TaxR (n=2), respectively.

*Expression of DEPTOR, RICTOR, RAPTOR, and mTOR*. Quantitative RT-PCR revealed that DEPTOR, RICTOR, RAPTOR, and mTOR are differentially expressed in paclitaxel-sensitive and -resistant (TaxR) ovarian cancer cell lines (n=3). There was an up-regulation of DEPTOR (2.4-fold), RICTOR (1.5-fold) and mTOR (1.2-fold) in PEO1TaxR ovarian cancer cells when compared with paclitaxel-sensitive PEO1 cells. A down-regulation (0.6-fold) was detected for RAPTOR (Figure 1A). With regards to SKOV-3, a significant up-regulation of DEPTOR (1.8-fold) and down-regulation of RAPTOR (0.6-fold), RICTOR (0.7-fold) and mTOR (0.6-fold) was found in SKOV-3TaxR cells when compared with parental SKOV-3 cells (Figure 2A).

*Protein expression and cellular distribution of mTOR*. The protein expression of mTOR was also assessed in these cell lines, using immunofluorescent analysis. A similar intensity in cytoplasmic staining was detected in both parental and PEO1TaxR cells. In SKOV-3 cells, the staining was more granular when compared with SKOV-3TaxR cells (Figure 1B). In PEO1 cells, no apparent differences in the intensity or localization of mTOR were observed for either parental or paclitaxel-resistant cells (Figure 2B).

*Expression p70S6K isoforms in paclitaxel-sensitive and TaxR cell lines*. Semi-quantitative RT-PCR analysis corrected with β-actin revealed that the expression of S6K α2 and β2 remained unaltered in SKOV-3 and PEO1 parental and paclitaxel-resistant ovarian carcinoma cell lines alike (Figure 3). Interestingly, gene expression of S6Kα1 and β1 altered. An up-regulation in the expression of S6Kα1 splice variant in PEO1TaxR cells at the mRNA level was noted when compared with control (paclitaxel-sensitive) PEO1 cells, whereas S6Kβ1 was almost non-detectable in PEO1TaxR cells. However, no apparent difference in the expression of these two variants was detected between parental SKOV-3 and SKOV-3TaxR cells (Figure 3A).

*Phosphorylation of mTOR and p70S6K in parental and TaxR cell lines*. The activity of mTOR and p70S6K was assessed by measuring the phosphorylation status of these two components. Under basal conditions (*i.e.* no treatments), there was no apparent difference in the phosphorylation of mTOR in Ser2448 in any of samples tested. Interestingly, a notable down-regulation in the phosphorylation of Ser371 and Thr389 p70S6K was noted in both SKOV-3 and SKOV-3TaxR compared with PEO1 parental and PEO1TaxR cells (Figure 3B).

## Discussion

This study used two cell lines that have been developed to study ovarian cancer drug resistance and signalling *in vitro* (27). In particular, SKOV-3 cells have been used extensively to dissect signalling pathways involved in the development of epithelial ovarian cancer. The sensitivity of PEO1 and PEO1TaxR cells to the cytotoxic effects of rapamycin were determined, but no differences were ascertained. Nevertheless, the effectiveness of rapamycin in MDR cell lines has been implicated in this study. This manuscript presents novel data on DEPTOR, a newly described modulator of mTOR signalling as highlighted by an altered expression in drug-resistant cells. The expression of DEPTOR was up-regulated in both PEO1TaxR and SKOV-3TaxR cells when compared with parental paclitaxel lines. A recent study shows low expression of DEPTOR in most types of cancer; however, it is highly overexpressed in a

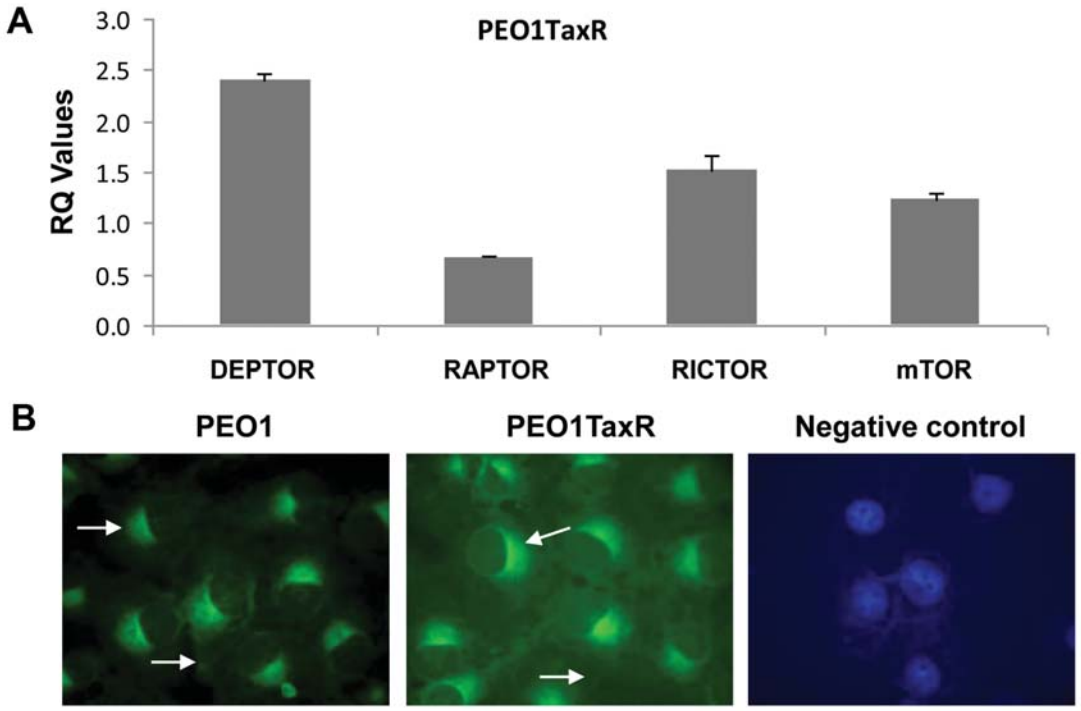


Figure 1. *A: Quantitative RT-PCR analysis of DEPTOR, RAPTOR and RICTOR and mTOR in PEO1 Parental and PEO1TaxR cells. B: Indirect immunofluorescent analysis for mTOR in PEO1 (PEO1 parental and PEO1TaxR) cells showing the cytoplasmic distribution of mTOR (white arrows). No apparent difference in mTOR protein expression between the two cell lines was noted. Negative serum controls confirmed specificity. The cell nuclei were visualised with the DNA-specific dye DAPI.*

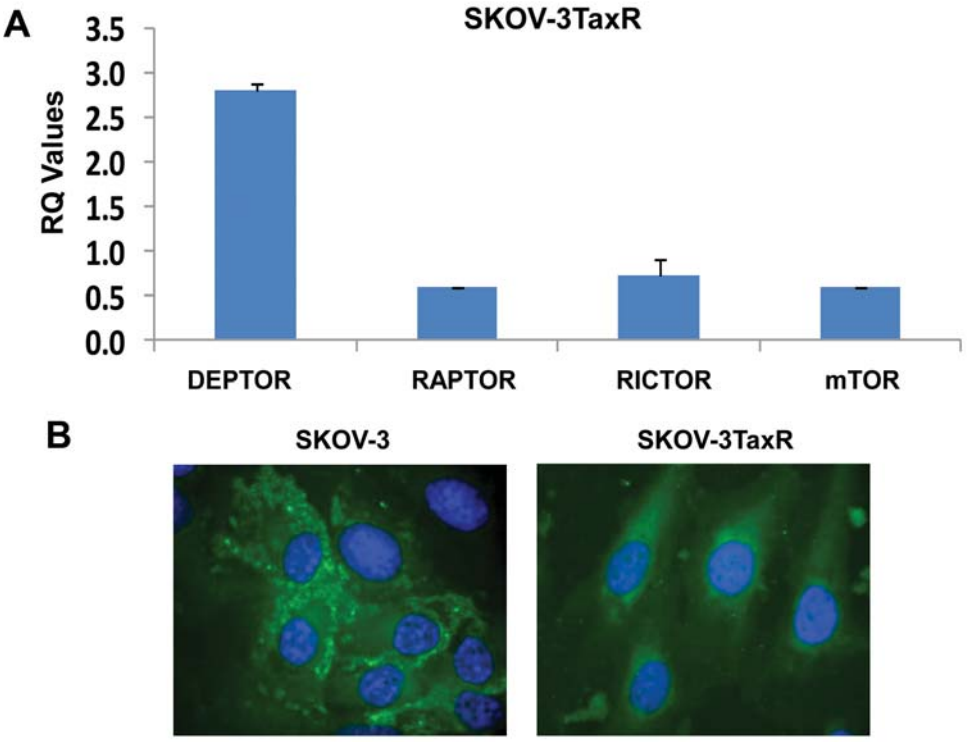


Figure 2. *A: Quantitative RT-PCR analysis revealed up-regulation of DEPTOR and down-regulation of mTOR, rector and RAPTOR in SKOV-3 parental cells when compared with paclitaxel-resistant cells. B: Immunofluorescent analysis for mTOR in SKOV-3 (SKOV-3 parental and SKOV-3TaxR) cells revealed cytoplasmic distribution of mTOR.*

subset of multiple myelomas with cyclin D1/D3 or c-MAF/MAFB translocations (23). The authors concluded that the increased presence of DEPTOR in these cells drives the activation of PI3K/Akt signalling cascade, promoting cell survival. It is possible that in a state of drug resistance, amplification of DEPTOR may lead to an increase in cell proliferation and promote cancer progression through increased survival.

These data also suggest that expression of mTOR signalling components may be cell type specific. For instance, RICTOR and mTOR expression were up-regulated in the PEO1TaxR cells, whereas their expression was markedly down-regulated in SKOV-3TaxR ovarian cancer cells. These data imply a higher order of complexity in the cross-talk between mTOR and its interacting protein DEPTOR. This differential expression of mTOR signalling components in these cell lines could be due to distinct characteristics of each cell line. For example, SKOV-3 cells do not express p53 and inhibition of mTOR activity with rapamycin resulted in $G_1$ arrest in SKOV-3 cells but not in OVCAR4 or OVCAR5 cells (16). These distinct characteristics suggest that each ovarian cancer cell line might differ with regards to their ability to respond to drug resistance. Consequently, downstream signalling cascades including mTOR could also be differentially regulated. Cellular localization of mTOR may have important implications for expression and consequently cancer treatment. mTOR protein is localised within the cytoplasm in both ovarian cancer cell lines. These data are in agreement with previous studies demonstrating the cytoplasmic localization of mTOR (28). Another study argued that mTOR can also shuttle between the nucleus and cytoplasm, an event necessary for the maximal activation of S6K1 (29); however, we did not detect nuclear localisation of mTOR in the cell lines used in this study. Interestingly, cytoplasmic mTOR protein expression was lower in the TaxR SKOV-3 cells when compared with paclitaxel-sensitive cells, in agreement with the quantitative RT-PCR analysis. Future studies could determine the cellular localisation of mTOR in ovarian tumours before and during drug treatment. Moreover, it will be interesting to map the cellular distribution of DEPTOR in ovarian tumours upon availability of a commercially available antibody. The activity of mTOR and p70S6K was assessed by measuring changes in the phosphorylation of these key kinases. Our data indicate that phosphorylation of these enzymes is differentially regulated in the ovarian cancer cell lines, suggesting further cell type-specific effects. This is particularly evident in SKOV-3 cells, where an association is evident between the dephosphorylation of p70S6K at Ser371 and Thr389. This concurs with studies showing that Ser371 phosphorylation regulates the phosphorylation status of Thr389 and directly influences S6K activity (30).

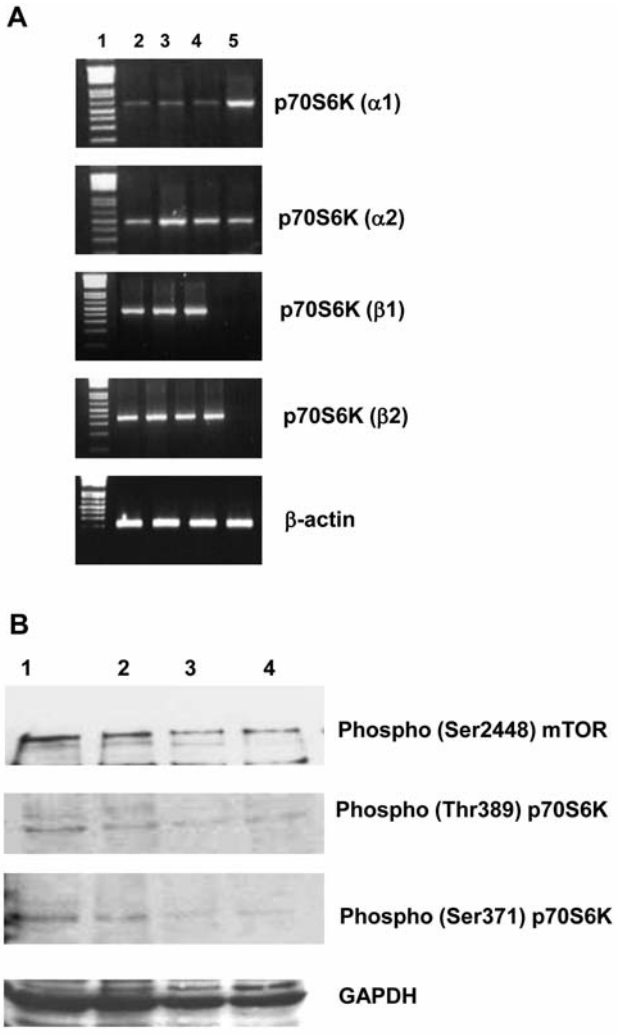


Figure 3. *A: Semi-quantitative RT-PCR analysis of mTOR and p70S6K variants. Lane 1: DNA marker, lane 2: cDNA from SKOV-3 parental cells, lane 3: cDNA from PEO1 parental cells, lane 4: cDNA from SKOV-3TaxR cells, and lane 5: cDNA from PEO1TaxR cells. B: Western blot depicting changes in the phosphorylation status of p70S6K and mTOR under basal conditions in paclitaxel parental and TaxR ovarian carcinoma cell lines, when compared with GAPDH. Lane 1: lysates from PEO1 parental cells, lane 2: lysates from PEO1TaxR cells, lane 3: lysates from SKOV-3 parental cells, and lane 4: lysates from SKOV-3TaxR cells.*

Collectively these novel data provide evidence that human ovarian cancer cell lines express mTOR and its downstream signalling components. Indeed paclitaxel resistance can affect gene expression and activity of these components, emphasising the significance of this pathway in both tumour development and treatment. The implications of the mTOR signalling pathway in the development of paclitaxel resistance in ovarian cancer may be paramount in determining future strategies to overcome this.

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