SPANDIDOS PUBLICATIONS

Activation of mTOR in a subgroup of ovarian carcinomas: Correlation with p-eIF-4E and prognosis

AURELIA NOSKE[1], JULIANE LENA LINDENBERG[1], SILVIA DARB-ESFAHANI[1], WILKO WEICHERT[1], ANN-CHRISTIN BUCKENDAHL[1], ANNIKA RÖSKE[1], JALID SEHOULI[2], MANFRED DIETEL[1] and CARSTEN DENKERT[1]

[1]Institute of Pathology, [2]Department of Gynecology and Obstetrics, Charité University Hospital Berlin, D-10117 Berlin, Germany

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Abstract. Ovarian carcinoma patients have an extremely poor prognosis; therefore, new molecular therapeutic approaches are urgently needed. The mTOR pathway, which may be targeted by substances such as Rapamycin or RAD001, is emerging as a promising target for anticancer therapy. So far, the expression and prognostic impact of mTOR signalling elements have not been completely studied in ovarian tumors. We analyzed p-mTOR, p-4E-BP1 and p-eIF-4E in 107 human ovarian lesions and observed an overexpression of p-mTOR (47%) and p-eIF-4E (56%) protein in primary ovarian carcinomas as compared to borderline tumors. Phospho-mTOR expression was significantly related to p-eIF-4E ($p \leq 0.001$) and serous histological type ($p=0.03$). Increased p-4E-BP1 (31%) was associated with poor differentiation ($p=0.04$) and higher mitotic rate ($p=0.004$). In univariate analysis, increased expression of p-mTOR and p-eIF-4E was significantly associated with better overall survival ($p=0.003$, $p=0.029$). To connect the expression data with mechanistic studies, a set of 10 ovarian cancer cell lines was used. Expression of p-mTOR was increased in all cancer cell lines as compared to ovarian surface epithelial (HOSE) cells. Rapamycin treatment revealed a reduction of p-mTOR and p-4E-BP1 but increased p-AKT levels. We show for the first time an association of p-mTOR and p-eIF-4E with better overall survival for ovarian cancer patients. The combined results of our *in vivo* and cell culture studies suggest that a subpopulation of these patients may benefit from mTOR inhibition. The design of future clinical trials should incorporate biomarker testing to determine predictive markers for response to mTOR inhibitors.

Correspondence to: Dr Aurelia Noske, Institute of Pathology, University Hospital Charité, Campus Mitte, Charitéplatz 1, D-10117 Berlin, Germany
E-mail: aurelia.noske@charite.de

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Introduction

Ovarian cancer is associated with the highest mortality among gynecologic malignancies, which is due to the lack of early clinical symptoms on the one hand, but due to the resistance to conventional chemotherapeutic approaches, on the other. Therefore, it is a necessity to identify molecular targets for novel therapies. In the setting of translational research in clinical trials, it is important to develop assays for biomarkers for prediction of therapy response, planning of individualized treatment concepts and estimation of patient prognosis.

The 289-kDa Ser/Thr kinase mTOR (mammalian target of Rapamycin) belongs to the phosphatidylinositol 3-kinase related kinase (PIKK) family of kinases. mTOR is affected by numerous cellular stressors, cell growth regulators and negatively regulated by tuberous sclerosis complex (TSC1, TSC2) gene products (1-3). Two functionally distinct TOR complexes exist in mammals, mTORC1 (Rapamycin-sensitive complex) and mTORC2 (Rapamycin-insensitive complex). mTORC1 [consisting of mTOR, mLST8 (GßL) and Raptor] controls mRNA translation and cell growth by phosphorylation of two major targets such as ribosomal S6 kinase 1 (S6K1) and eukaryotic translation inhibition factor 4E binding protein (4E-BP) family (4). Phosphorylation of 4E-BP1 results in the release of the cap-binding phosphoprotein eukaryotic initiation factor 4E (eIF-4E) allowing the formation of an eIF-4F complex (consisting of eIF-4E, eIF-4A, eIF-4G) to facilitate ribosome loading and mRNA translation (5). So far, the functional significance of eIF-4E phosphorylation has not been completely determined.

mTORC2 (consisting of mTOR, mLST8 and Rictor) regulates actin cytoskeleton dynamics. It has been shown that mTORC2 is part of the AKT activating pathway by direct phosphorylation of AKT on Ser473 (6). AKT activates mTOR and is regulated by mTOR through both positive and negative feedback loops (2).

These data point to the mTOR pathway as an interesting biological target. Compared with other pathways, a further advantage is that substances that target mTOR are already available in the clinical setting. The mTOR inhibitor Rapamycin leads to dephosporylation of 4E-BP1 and p70S6K

resulting in inhibition of translation initiation and cell proliferation. Rapamycin and its analogues (CCI-779, RAD001) have shown promising anti-tumor activity such as for advanced renal cell and breast cancer in clinical phase II and III trials (7,8), but to our knowledge there are no ongoing trials in ovarian cancer yet. As a basis for clinical trials, it is necessary to identify patients who might have a benefit of this therapy and to determine molecular biomarkers that can be used in tumor tissue to investigate the status of the target molecular pathway. Recent cell culture data suggest that mTOR inhibitors may be active against ovarian cancer cells (9). Until now however, the protein expression pattern as well as the prognostic impact of elements of the mTOR cascade have not been completely studied in human ovarian tumors *in vivo*.

We investigated the expression pattern of p-mTOR, p-4E-BP1 and p-eIF-4E in primary human ovarian carcinomas in comparison with borderline tumors and benign ovarian tissue. Furthermore, we analyzed the relationship between these molecules and compared expression data with clinicopathological characteristics and patient outcome. To link the *in vivo* data with functional mechanistic studies, we determined the effect of inhibition of mTOR by Rapamycin on protein expression as well as cell proliferation.

Materials and methods

Study population and tissue samples. Immunohistochemical examination was performed retrospectively on tissue samples taken for routine diagnostic and therapeutic purposes. Normal ovarian tissue, benign and malignant ovarian tumors of 107 patients who were diagnosed at the Institute of Pathology, Charité Hospital Berlin between 1989 and 2003 were included in the study. The tissue specimens consisted of 83 primary invasive ovarian carcinomas, 14 borderline tumors and 10 benign lesions. Tissue samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Routine hematoxylin and eosin sections were performed for histopathological evaluation. The stage of tumors was assessed according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. For grading of tumors the Silverberg grading system evaluating architectural, nuclear and mitotic features was used (10). The number of mitoses per 10 high power fields (hpf) was evaluated and separated in three groups, <9/10 hpf, 10-23/10 hpf and >24/10 hpf. The median follow-up time of the surviving patients was 39 months. Characteristics of the study population are summarized in Table I.

Table I. Characteristics of 83 patients with invasive ovarian carcinomas.

Characteristics	All cases (%)
Age at surgery (years)	
≤60	45 (54.2)
>60	38 (45.8)
Histological type	
Serous	53 (63.9)
Non-serous	23 (27.7)
Undifferentiated	7 (8.4)
FIGO stage	
I	16 (19.3)
II	9 (10.8)
III	53 (63.9)
IV	5 (6.0)
Tumor stage	
pT1	18 (21.7)
pT2	10 (12.0)
pT3	55 (66.3)
Nodal stage (n=67)	
pN0	41 (61.2)
pN1	26 (38.8)
Tumor grade	
G1	10 (12.0)
G2	36 (43.4)
G3	37 (44.6)
Intraoperative residual tumor (n=51)	
Residual tumor <2 cm	44 (86.3)
Residual tumor ≥2 cm	7 (13.7)
Chemotherapy (n=69)	
Platinum-based	63 (91.3)
Non-platinum	2 (2.9)
No chemotherapy	4 (5.8)

Antibodies. Primary antibodies were polyclonal against p-AKT (Ser473), p-4E-BP1 (Thr37/46), p-eIF-4E (Ser209) as well as monoclonal against p-mTOR (Ser2448) (Cell Signaling Technology, Inc., Beverly, MA).

Immunohistochemistry. Immunohistochemical examination was performed on tissue microarrays. Therefore, representative areas of the ovarian carcinomas as well as borderline tumors were marked on the routine H&E stained histological sections. Four tissue cores of 1.5 mm diameter from different tumor parts of the donor blocks were punched using a tissue micro-arrayer (Beecher Instruments, Woodland, USA) and positioned in a recipient paraffin array block. Immunohistochemistry was performed according to standard procedures. Briefly, slides were boiled in citrate buffer (pH 6.0) in a pressure cooker for 5 min and incubated with primary antibody for 1 h at room temperature. Primary antibodies included p-mTOR, p-4E-BP1 and p-eIF-4E in a dilution of 1:100. Immunostaining was followed by incubation with EnVision detection system (Dako) and visualized using DAB (diaminobenzidine) chromogen solution. The immunostaining was evaluated independently by two pathologists (A.N., C.D.), who were blinded towards patient outcome. Cases with disagreement were discussed at a multihead microscope. The expression of p-mTOR and p-4E-BP1 was evaluated according to the percentage of positive cells and the intensity of staining. The percentage of positive cells was scored as: 0 (0%); 1 (<10%); 2 (11-50%); 3 (51-80%); 4 (>80%). The

ntensity was scored as: 0 (negative), 1 (weak), 2 e) and 3 (strong). For the immunoreactive score (IRS) the percentage of positive cells and staining intensity was multiplied, resulting in a value between 0 and 12. To separate cases with a weak or a strong immunoreaction in tumor tissue, we combined cases for p-mTOR with an IRS of 0-3 to one group with negative to weak expression ('negative'), while cases with an IRS of 4-12 were considered as 'positive'. Since p-4E-BP1 was found in the nucleus and cytoplasm, we performed a separate evaluation. For both expression patterns, the cut-off point of dichotomization of the immunoreactive score was 6. Immunostaining of p-eIF-4E showed a weak intensity, therefore we estimated only the percentage of positive tumor cells and classified the tumors as positive (>50% of cells stained) or negative. Negative controls were performed by omitting the primary antibody. The antibody specifity was evaluated by Western blotting.

Statistical analysis of expression data. The statistical significance of the association between p-mTOR, p-4E-BP1 and p-eIF-4E expression and clinicopathological parameters was assessed by χ^2 test for trends or Fisher's exact test. The probability of overall survival as a function of time was determined by the Kaplan-Meier method. For survival analysis only the patients with invasive carcinomas were included. Differences in survival curves were compared by the log-rank test. Multivariate survival analysis was performed using the Cox model of proportional hazards. P-values <0.05 were considered as significant. For the statistical evaluation the SPSS software Version 13.0 was used.

Cell culture and inhibitors. The human ovarian carcinoma cell lines (OVCAR-3, SKOV-3, CAOV-3, ES-2, A27/80, Mdah2744, OAW42) investigated in this study have been previously described (11,12). EFO21, EFO27, FU-OV-1 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Heidelberg, Germany). Cells were cultured in DMEM (Bio Whittaker Europe, Verviers, Belgium) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria). The HOSE cells (13), an immortalised human ovarian surface epithelium cell line, were cultivated in a 1:1 mixture of medium 199 (Sigma Chemicals Co., St. Louis, MO) and MCDB 105 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum and 2 mM glutamine. All cell lines were maintained at 37˚C in a humidified atmosphere of 5% CO_2/95% air.

We used the mTOR inhibitor Rapamycin (Cell Signaling Technology, Inc.) in different concentrations (20-1000 nM) for 48 and 72 h as well as for 6 and 8 days. The inhibitor was dissolved in DMSO, which was used as a solvent control in control experiments.

Immunoblotting. For protein analysis, cells were lysed in 100 μl of 62.5 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol, 50 mM DTT and 0.1% bromophenol blue. Protein/sample (100 μg) were separated on a polyacrylamide gel (6-14%), blotted onto nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany), washed in PBS, and blocked in buffer [1X PBS, 0.1% Tween-20, 5% I-block (Tropix, Bedford, MA, USA)] for 1 h at room temperature. Membranes were probed with antibodies against p-AKT, p-mTOR, p-4E-BP1 and eIF-4E overnight at 4˚C, diluted 1:1000 in blocking buffer, and followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Tropix). Bands were visualized using the CDP star RTU luminescence system (Tropix).

Cell proliferation assay. To examine the effect of Rapamycin on cell proliferation, we incubated OVCAR-3, SKOV-3 and A27/80 cells (plated in 96-well plates; 3000 cells/well) for 72 h, 6 and 8 days with Rapamycin (20, 100 and 500 nM). Cell number was determined by using an XTT-based colorimetric assay (Roche Diagnostics, Germany) according to the manufacturer's instructions. All experiments were performed in triplicates. Differences in cell proliferation were evaluated statistically by the two-sided t-test.

Cell cycle analysis and apoptosis assay. For cell cycle analysis, OVCAR-3, SKOV-3 and A27/80 were plated at $4x10^5$ cells/well in 6-well plates. After 24 h, cells were treated with Rapamycin at different concentrations (20-1000 nM) for 48 and 72 h as well as for 6 and 8 days. DMSO served as a solvent control. Following, cells were fixed in 70% ice cold ethanol at -20˚C for 30 min. The percentage of cells in G0/G1, S and G2/M phase was quantified by using a propidium iodide-based cellular DNA flow cytometry analysis method. Briefly, cells were rinsed in PBS and 500 μl diluent buffer (50 μl of 0.1% Triton X-100, 250 mg of 0.5% BSA in 50 ml PBS) was added. RNAse digestion was carried out by adding 4 μl RNAse (10 mg/ml) for 1 h at 37˚C. Then, cells were centrifuged and diluted in 500 μl diluent buffer containing 20 μl propidium iodide and evaluated by fluorescence activated cell sorting in a FACScan (BD Biosciences, Heidelberg, Germany). Experiments were performed in three separate experiments. Analyses of cell cycle data were done with Cyclchred and WinMDI (Freeware).

OVCAR-3, SKOV-3 and A27/80 cells were also evaluated for evidence of apoptosis by measurement of the DNA content by propidium iodide (PI) staining and annexin V binding. Briefly, cells were seeded at a density of $4x10^5$ cells/well in 6-well plates and treated with Rapamycin as described above. After 48 h, cells and supernatants were collected together, centrifuged at 2000 rpm for 5 min. The pellet was incubated with 100 μl binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$ in 50 ml H_2O), 2.5 μl Annexin V-FITC (Roche) and 5 μl propidium iodide. After 15 min, 300 μl binding buffer was added. Cells were imme-diately measured with the FACScan flow cytometer (BD Biosciences).

Results

Increased expression of p-mTOR and p-eIF-4E in ovarian carcinomas compared to borderline tumors. To investigate key elements of the mTOR pathway in ovarian lesions, we analyzed protein expression by immunohistochemistry. We found a cytoplasmic expression pattern of p-mTOR in 39 (47%) out of 83 ovarian carcinomas. In contrast, in borderline tumors, benign ovarian lesions and normal ovarian epithelium only weak or no expression was detected. For p-4E-BP1, we

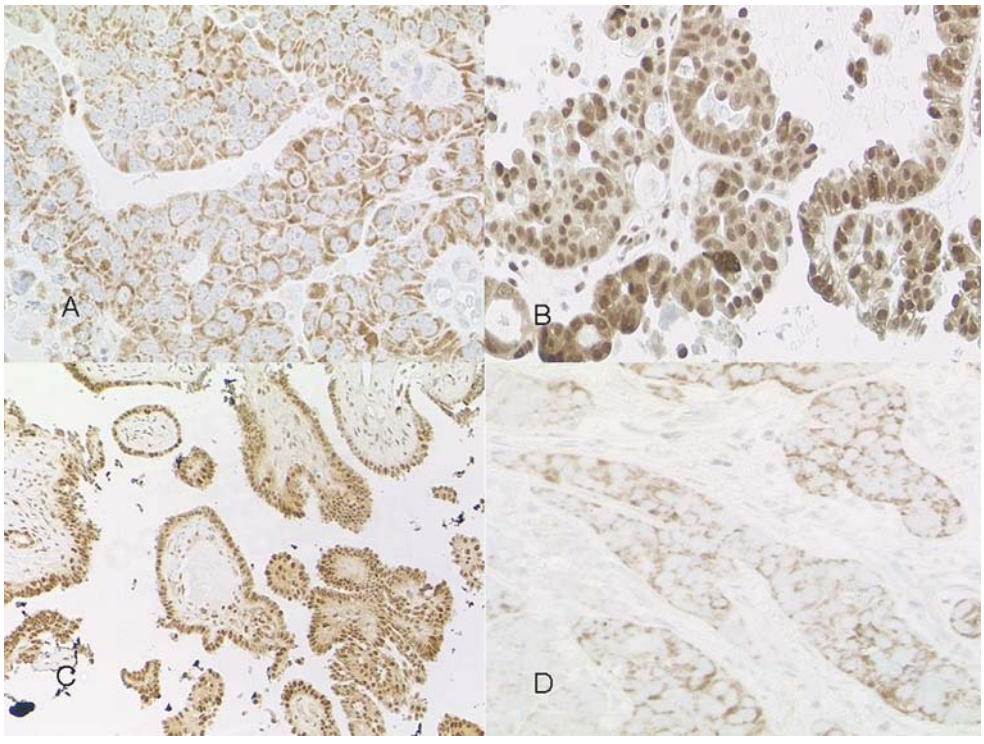

Figure 1. Immunohistochemical expression analysis of p-mTOR, p-4E-BP1 and p-eIF-4E in invasive carcinomas and borderline tumors of the ovary. (A) Strong cytoplasmic expression of p-mTOR in a high-grade serous carcinoma. (B) Strong nuclear and weak cytoplasmic staining of p-4E-BP1 in a moderately differentiated carcinoma. (C) Strong nuclear expression of p-4E-BP1 in the epithelium of a serous borderline tumor. (D) Strong cytoplasmic immunostaining of p-eIF-4E in a high-grade serous ovarian cancer.

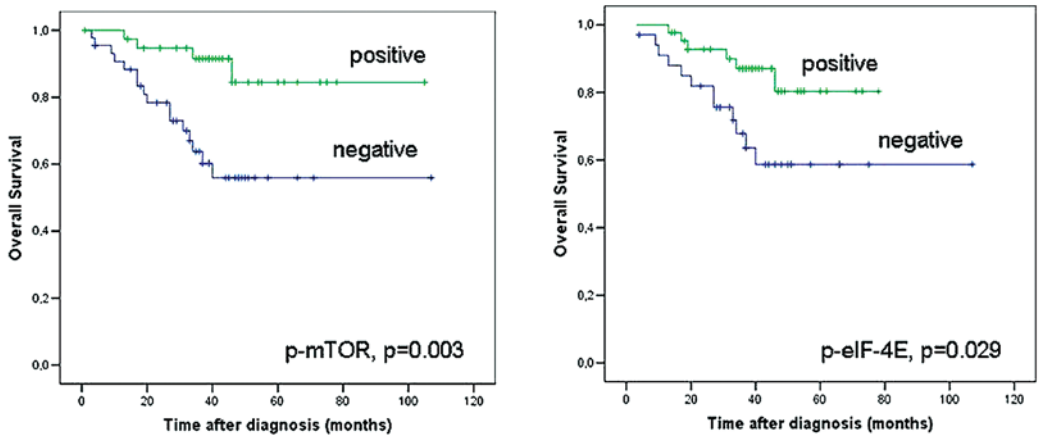

Figure 2. Univariate Kaplan-Meier survival analyses according to expression of p-mTOR and p-eIF-4E displaying significant better overall survival in patients with ovarian carcinomas.

observed a nuclear expression in 25 (30.5%) and a cytoplasmic expression in 12 (14.6%) out of 82 ovarian carcinomas. Furthermore, we found a nuclear expression in 7 (50%) out of 14 borderline tumors as well as in 8 (80%) of 10 benign ovarian lesions.

For p-eIF-4E, an expression in 44 out of 79 (55.7%) carcinomas was found, whereas borderline tumors, benign ovarian lesions and normal ovarian epithelium displayed no expression. Representative images are shown in Fig. 1.

Expression of p-mTOR correlates with p-eIF-4E. To evaluate the relationship between different elements of the mTOR pathway in tumor tissue, we correlated the relevant biomarkers with each other. Interestingly, we found that p-mTOR expression was significantly associated with p-eIF-4E expression ($p \leq 0.001$).

Expression of p-mTOR and p-eIF-4E correlates with prognosis. The immunohistochemical analysis revealed a subgroup of tumors with an overexpression of elements of the mTOR signaling pathway. To further characterize this subgroup, we have studied classical clinicopathological parameters. The increased p-mTOR expression was significantly associated with a serous histological type (p=0.03). Nuclear

Correlation of p-mTOR expression with pathological features.

Characteristics	All cases n (%)	Phospho-mTOR n (%)		P-value
		Negative	Positive	
All carcinomas	83 (100)	44 (53)	39 (47)	
p-eIF-4E (n=79)				0.000
Negative	35 (100)	28 (80)	7 (20)	
Positive	44 (100)	15 (34.1)	29 (65.9)	
Histological type				0.030
Serous	53 (100)	23 (43.4)	30 (56.6)	
Non-serous	23 (100)	16 (69.6)	7 (30.4)	
Undifferentiated	7 (100)	5 (71.4)	2 (28.6)	
FIGO stage				0.722
I-II	25 (100)	14 (56)	11 (44)	
III-IV	58 (100)	30 (51.7)	28 (48.3)	
Tumor stage				0.192
pT1	18 (100)	12 (66.7)	6 (33.3)	
pT2+3	65 (100)	32 (49.2)	33 (50.8)	
Nodal stage (n=67)				0.163
0	41 (100)	18 (44)	23 (56)	
1	26 (100)	16 (61.5)	10 (38.5)	
Tumor grade				0.294
1+2	46 (100)	22 (47.8)	24 (52.2)	
3	37 (100)	22 (59.5)	15 (40.5)	

p-4E-BP1 was significantly related to poorly differentiated carcinomas (p=0.04) and higher mitotic rate (p=0.004). Statistical results are given in Table II. Expression of cytoplasmic p-4E-BP1 and p-eIF-4E were not significantly associated with other clinicopathological features (data not shown).

In univariate Kaplan-Meier analysis, increased expression of p-mTOR or p-eIF-4E was related to a better overall survival for patients with invasive ovarian cancer (p=0.003 and p=0.029), shown in Fig. 2. Phospho-4E-BP1 was not associated with patient survival. In an exploratory multivariate analysis that included histological grade, tumor stage, postoperative residual tumor, age and p-mTOR or p-eIF-4E expression, only residual tumor size had significant independent prognostic information.

Expression of p-AKT, p-mTOR, and p-4E-BP1 in ovarian cancer cells. In the next steps we connected the *in vivo* data of mTOR pathway proteins with mechanistic cell culture studies. We investigated the expression and activation status of phospho-AKT, phospho-mTOR and phospho-4E-BP1 in 10 ovarian cancer cell lines as well as in immortalized human ovarian surface epithelium cells (HOSE) by Western blotting. All ovarian cancer cell lines had an increased expression of p-AKT, p-mTOR, and p-4E-BP1 as compared to HOSE cells. We further found different protein expression patterns between the ovarian cancer cell lines indicating a distinct activation state of these molecules (Fig. 3). Thus, increased p-AKT was observed in OVCAR-3, SKOV-3, A27/80, and EFO-21 cells, while p-mTOR was expressed in all investigated ovarian cell lines with a particular strong expression in OVCAR-3, SKOV-3 and CaOV-3.

Rapamycin inhibits cell proliferation of ovarian cancer cell lines. We incubated OVCAR-3, SKOV-3, A27/80, Mdah2774 and OAW-42 with Rapamycin to investigate the effect on cell proliferation by XTT-assay. To assess short- and long-term effects of Rapamycin on the protein expression and activation, we used different concentrations and incubation times to evaluate the optimal effect of this agent. The ovarian cancer cells were treated with different concentrations (20, 100, 500 nM). Measurements were performed after 72 h, 6 and 8 days. DMSO served as a solvent control. We observed a significant inhibiton of cell proliferation in OVCAR-3 cells as compared to the control after 72 h (p=0.004, t-test) and 6 days (p=0.018). The inhibition of cell proliferation did not depend on the concentration of Rapamycin. Thus, concentrations of 20 nM resulted in growth inhibition from 100% to approximately 63% after 72 h and 73% after 6 days. Similar results were observed in SKOV-3 (72 h, p=0.019; 6 days, p=0.019) and A27/80 cells (72 h, p=0.005; 6 days, p=0.021).

Representative results are shown in Fig. 4. In contrast, OAW-42 (p-4E-BP1 negative) and Mdah2774 (p-AKT-

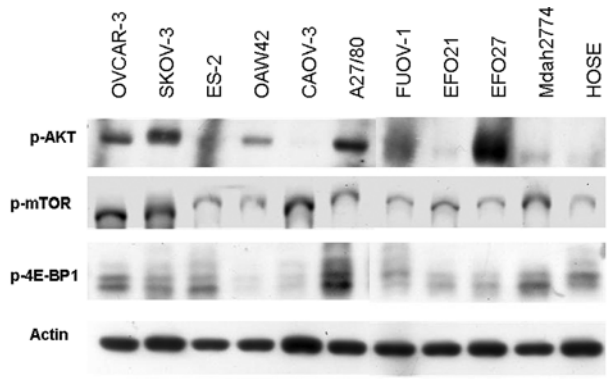

Figure 3. Expression of p-AKT, p-mTOR, p-4E-BP1 and p-eIF-4E on protein level in ovarian carcinoma cell lines and HOSE cells investigated by immunoblotting. Actin served as loading control. One of three independent experiments is shown.

negative) cells showed no significant reduced cell proliferation (data not shown).

Effects of Rapamycin on expression of pAKT, p-mTOR and p-4E-BP1, in ovarian cancer cells. To investigate the influence of Rapamycin on the protein expression of members of the mTOR cascade, we investigated three ovarian cancer cell lines (OVCAR-3, SKOV-3, A27/80), which had shown a strong inhibitory effect of Rapamycin on cell proliferation. The cells were incubated with Rapamycin for 48 h in different concentrations (20-1000 nM). Rapamycin treatment strongly induced p-AKT expression in a concentration-independent manner in all three cell lines, whereas decreased expression levels of p-mTOR and p-4E-BP1 were found. Representative data are shown in Fig. 5.

Effects of Rapamycin on cell cycle and apoptosis. Ovarian cancer cells (OVCAR-3, SKOV-3, A27/80) were treated with Rapamycin and the cell cycle distribution was analyzed by flow cytometry. No significant cell cycle arrest was found. Further, we did not observe an induction of apotosis in these Rapamycin-treated cancer cells using annexin-V/propidium iodide staining and FACS analysis (data not shown).

Discussion

In this study, we investigated the hypothesis that the mTOR pathway might be dysregulated in human ovarian cancer. In line with this hypothesis, we observed i) expression of p-mTOR and its up- and downstream molecules in a subpopulation of ovarian carcinomas as well as different expression profiles in ovarian cancer cell lines, ii) improved overall survival for patients with p-mTOR and p-eIF-4E positive carcinomas, and iii) inhibition of the cell proliferation by Rapamycin. The data support the importance of this signaling pathway in ovarian carcinomas and suggest that mTOR inhibition may be effective in a subset of tumors.

Expression and relationship of p-mTOR, p-4E-BP1 and p-eIF-4E. We found an increased protein expression of p-mTOR in 47% of ovarian carcinomas and a significant association with p-eIF-4E as well as serous tumor differentiation. Elevated expression of p-mTOR was previously found in 17 out of 31 (55%) ovarian carcinomas as analyzed by immunohistochemistry (14). Apart from these findings, data on p-mTOR

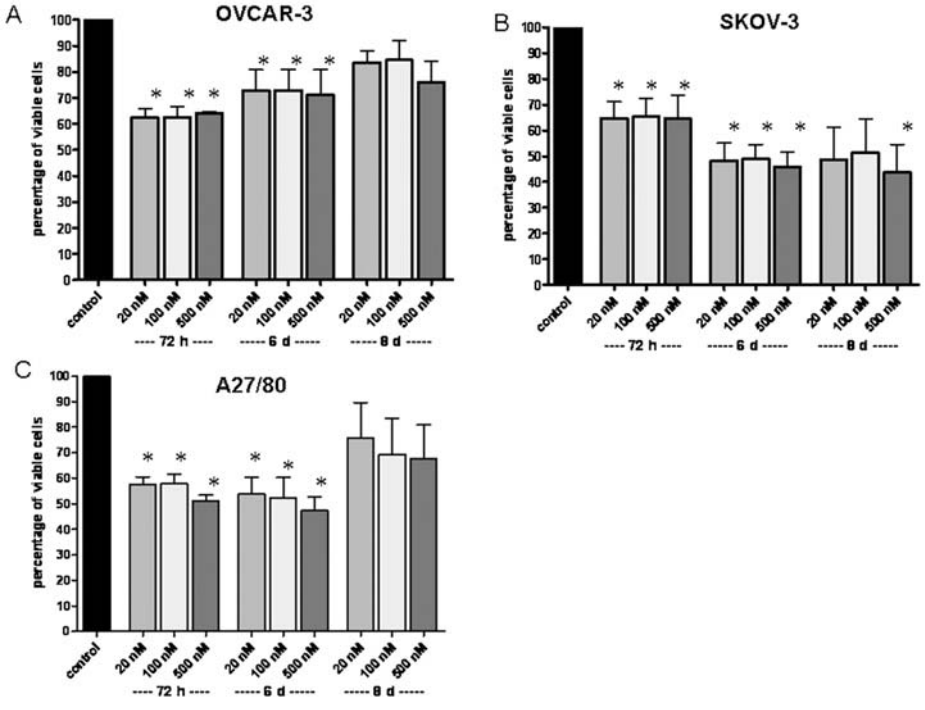

Figure 4. Effects of Rapamycin in ovarian cancer cells. (A-C) OVCAR-3, SKOV-3 and A27/80 cells were incubated with Rapamycin in different concentrations and incubation times and cell proliferation was measured using an XTT assay. Mean and SD of four measurements performed in triplicates after 72 h, 6 and 8 days are shown. *A significant difference in cell proliferation ($p<0.05$, two-sided t-test) was statistically determined. The control was set to 100%.

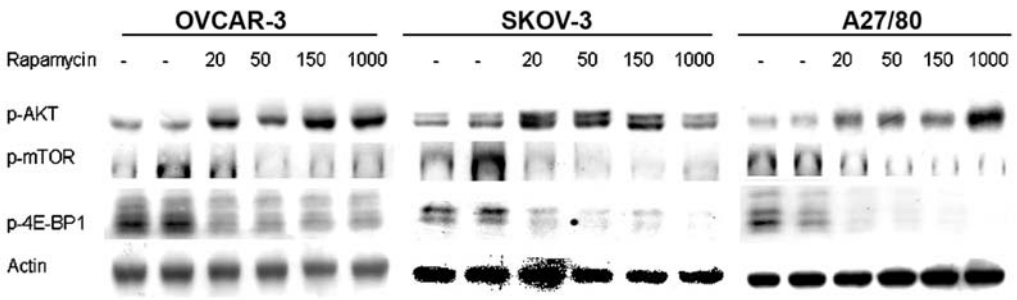

Figure 5. Effects of Rapamycin on protein expression. Phospho-AKT protein is overexpressed in OVCAR-3, SKOV-3 and A27/80 cancer cells after treatment with Rapamycin in dose-independent manner, while p-mTOR and p-4E-BP1 expression levels are reduced. DMSO served as a solvent control (second lane) and actin as loading control.

expression in ovarian cancer and its correlation to clinical and pathological features are rare. Increased expression of p-mTOR was further detected in 42% of invasive breast carcinomas (15) and 52% of advanced cervical carcinomas (16). To the best of our knowledge, we investigated for the first time phospho-eIF-4E in ovarian cancer by immunohistochemistry and found an expression in 56% of the invasive carcinomas. While overexpression of eIF-4E has been reported in various human carcinomas and cell lines (5) investigations of the phosphorylation state of eIF-4E in cancer are lacking. Further, we observed a nuclear expression of p-4E-BP1 in 31% of the ovarian carcinomas as well as an increased expression in the borderline tumors and benign ovarian lesions. The expression was associated with high-grade carcinomas and high mitotic frequence which is in line with a recent study of Castellvi *et al* (17).

Relation of p-mTOR and p-eIF-4E as well as patient survival. We found that the subgroup of ovarian carcinomas with an increased expression of p-mTOR had a significantly better overall survival in univariate analysis. Data on p-mTOR expression and its correlation to ovarian cancer patient outcome are still lacking. Recently, expression of p-4E-BP1 (Thr70) was associated with a poor prognosis in ovarian cancer patients (17). Similarly, p-4E-BP1 was related to high-grade and high-stage breast carcinomas (18). Invasive breast carcinomas with high expression levels of p-mTOR and p-AKT were associated with shorter disease-free survival (15). It is known from own and other studies that elevated AKT is associated with poorly-differentiated and advanced ovarian carcinomas as well as poor prognosis (12,19-21), but a correlation of p-AKT and p-mTOR was demonstrated only in a small cohort of ovarian tumors by immunohistochemistry (14). Cell biological studies have shown a direct linkage between mTOR and the AKT-pathway and a regulation through positive and negative feedback loops (2). Thus, AKT activates mTOR and mTORC2 activates AKT suggesting a common or simultaneous activity and function in tumor cells. Therefore it is conceivable that enhanced mTOR activity might suppress functions of AKT resulting in tumor growth inhibition and better patient prognosis. Supporting this hypothesis, we found a different expression profile of p-AKT and p-mTOR using several ovarian cancer cell lines. While phospho-AKT was present only in four out of 10 cancer cell lines, phospho-mTOR was expressed nearly in all cancer cells. Phospho-4E-BP1, a major mTOR target was expressed in both p-AKT positive and negative cells. These findings indicate that the mTOR signaling pathway is activated in ovarian cancer cell lines but activation of mTOR and its downstream targets is not only mediated by AKT.

We analyzed for the first time the expression of phospho-eIF-4E, a molecule downstream of mTOR, in ovarian cancer and found a correlation with p-mTOR expression as well as better overall survival. Upon phosphorylation of 4E-BP1 (not only via AKT/mTOR), eIF-4E is released and the translation initiation is enhanced. While increased eIF-4E expression is related to metastatic progression and suppression of apoptosis (5), the functional significance of eIF-4E phosphorylation is not completely clear. Earlier studies have shown a high correlation of phospho-eIF-4E with protein synthesis (22,23), but a recent report indicates that p-eIF-4E is associated with a decrease of the overall protein synthesis rates during *in vitro* maturation of pig oocytes (24).

Effects of Rapamycin in ovarian cancer cells. Since the mTOR inhibitor Rapamycin and its analogs entered clinical trials suggesting functioning as a promising anticancer agent, it would be interesting to know which tumors are susceptible to this drug. Further, it would be helpful to identify biomarkers which predict the effects of Rapamycin. The mTOR inhibitors are in phase II and III trials on various carcinomas (25), but to our knowledge not in ovarian cancer. Recently, it has been shown that the mTOR inhibitor RAD001 delayed development and progression of ovarian cancer in transgenic mice (9). In line with previous studies we observed a concentration-independent inhibition of cell proliferation in Rapamycin treated ovarian cancer cell lines (26,27). We did not find a significant G1 cell cycle arrest as reported previously (14,27).

The effects of Rapamycin on the proteins of the mTOR cascade in ovarian cancer cells have not been widely examined. Here, we found an inhibition of p-mTOR and p-4E-BP1 expression and intense up-regulation of p-AKT in Rapamycin-treated ovarian cancer cells. Activation of p-AKT by Rapamycin was recently reported (25,28). In contrast to our findings; mTOR inhibition did not apparently change the p-mTOR levels in NSCLC cells (25), ErbB2-transfected breast cancer cells (15) and MOVCAR6 cells (9). Rapamycin binds directly to mTORC1 (Rapamycin-sensitive complex), inhibits phosphorylation of 4E-BP1 and activates

AKT (25,29). Since prolonged Rapamycin treatment revealed a cell type specific inhibition of mTORC2 (Rapamycin-insensitive complex) and AKT, the inhibition of mTORC1 and mTORC2 does not occur simultanously and AKT activity varies with the length and dosing of Rapamycin treatment (29). One possible explanation is that mTOR will be sequestered away from mTORC2 leading to decreased S473 phosphorylation and AKT activation (6). Further, it was reported that tumors with hyperactivated AKT are more sensitive to the effects of Rapamycin (14,30). Thus, Rapamycin effectively inhibits tumor growth when AKT signaling is the driving force in proliferation.

Sun *et al* reported for the first time an increased phosphorylation of eIF-4E in Rapamycin treated cancer cells (NSCLC) which may counteract the mTOR inhibitory effect, resulting in a decreased growth-inhibitory effect of Rapamycin (25). We tried to investigate expression of phosphorylated eIF-4E but did not achieve suitable results with commercial p-eIF-4E antibody in Western blotting. However, further investigations are needed to examine the p-eIF-4E status with suitable antibodies in cell culture. Since eIF-4E is released from 4E-BPs not only through PI3K/AKT- but also RAS-ERK-pathway (5), mTOR inhibitors can not repress the function completely.

We did not observe apoptosis or cell necrosis which is in line with other studies (27,31). However, effects of Rapamycin on apoptosis may depend on the cell type and genetic alterations such as lacking functional p53 or mutations in TSC2 (1,26,32). We suppose that in ovarian cancer cells Rapamycin prevents apoptosis by inhibition only of mTORC1 (and not mTORC2). Thus, mTORC1 inhibition leads to activation of AKT which promotes cell survival.

Our data indicate the importance of the mTOR pathway in ovarian carcinomas. Further, the findings support the view that mTOR inhibition may be effective in a subset of carcinomas that depend on AKT activity for survival, but not effective in all tumors that exhibit AKT activation (30). In addition, we conclude that the effects may depend on the phosphorylation state of eIF-4E. Rapamycin is unable to prevent and block tumor growth completely; therefore a combination with agents used in first line therapy would reveal synergistic effects (32). Moreover, a combination with a PI3K/AKT inhibitor (25,29) and/or MAPK inhibitor (9) might be a strategy to overcome the proliferative effects of (hyperactivated) AKT.

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