Expression of Phosphorylated Akt, mTOR and MAPK in Type I Endometrial Carcinoma: Clinical Significance

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Abstract. *Background/Aim: The Akt/mTOR and MAPK pathways are frequently activated in various tumor types but data on endometrial carcinoma are limited. The aim of the present study was to investigate the clinical significance of the expression of phosphorylated MAPK, Akt and mTOR (p-MAPK, p-Akt, p-mTOR) in type I endometrial carcinoma. Materials and Methods: The study comprised of 103 formalin-fixed paraffin-embedded (FFPE) type I endometrial carcinoma cases, retrospectively retrieved and assessed by immunohistochemistry for p-MAPK, p-Akt and p-mTOR expression. The expression of these proteins was also studied in non-neoplastic endometrial tissue adjacent to the tumor. Results: The expression patterns of these molecules differed between malignant and non-tumorous tissue specimens. The immunoreactivity for p-Akt was exclusively detected in the neoplastic tissues. Expression levels of p-MAPK were higher in tumors compared to non-neoplastic endometrium ($p<0.001$), while p-mTOR was found to be over-expressed in non-neoplastic endometrium compared to carcinomas ($p=0.001$). Expression of p-Akt was correlated with p-MAPK protein levels ($p=0.022$, $r=0.229$). On the other hand, no association was found with clinicopathological parameters and with disease-free (DFS) or overall survival (OS) of the patients. Conclusion: Our findings support the de-regulation of the PI3K/Akt/mTOR and MAPK signaling pathways in type I endometrial carcinomas suggesting involvement of these pivotal pathways in endometrial carcinogenesis.*

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Endometrial cancer is the most common malignancy of the female genital tract in developed countries. In the United States, 47,170 new cases of endometrial cancer were detected in 2012, leading to more than 8,000 deaths (1). In Europe, the incidence of endometrial cancer is rising over the last years because of the aging of the population, use of hormone replacement therapy and increasing prevalence of obesity (2).

In 1983, it was proposed that the majority of endometrial carcinomas could be divided into two different histological subtypes with distinct risk factors and clinical presentations (3), although certain endometrial carcinomas present with mixed characteristics (4). Type I endometrial carcinomas consist of endometrioid adenocarcinoma together with mucinous adenocarcinoma and account for 80-90% of cases of endometrial cancer. They arise through persistent, unopposed estrogen stimulation of the endometrium, frequently in a background of endometrial hyperplasia (5). The prognosis of this type of endometrial cancer, especially in its early stages, is favorable with a 5-year survival rate of 85-90% (6). Type II endometrial carcinoma includes serous carcinoma and clear cell carcinoma and accounts for 10-20% of endometrial cancers. It is unrelated to estrogen, arises in association with endometrial atrophy and has an aggressive clinical course, with early spread and poor outcome, as the 5-year survival rate ranges from 30% to 70%, even in the early stages (7).

Studies of endometrial carcinoma have highlighted the distinct molecular mechanisms participating in the pathogenesis of the two types of the disease (8). In type I endometrial carcinoma, the most frequent alterations involve *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) inactivation, alterations in *K-RAS*, catenin B1 (*CTNNB1*) and phosphatidyl-inositol-3-kinase catalytic subunit (*PIK3CA*) genes along with microsatellite instability (MSI). Type II endometrial carcinomas are frequently associated with *p53* mutations, *p16* inactivation, over-expression or amplification of *HER-2/neu* and E-cadherin (*CDH1*) inactivation (9).

The PI3K/Akt/mTOR pathway is one of the major signaling pathways that have been identified as important regulators of several critical cellular pathways and de-regulation of Akt has been associated with human malignancies (10). The PI3K/Akt/mTOR pathway is activated by binding of growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF) and insulin-like growth factor-1 (IGF-1) to their receptors, followed by receptor autophosphorylation and activation. The phosphoinositol-3-kinase (PI3K) is recruited to the intracellular segment of the receptor and triggers a series of events resulting in recruitment of Akt to the cell membrane and subsequent activation by phosphorylation. There are three Akt proteins. Akt-1 is primarily involved in induction of protein synthesis and inhibition of apoptosis, Akt-2 is involved in insulin signalling and Akt-3 is primarily expressed in the brain. Akt protein is activated by phosphorylation on two critical residues, serine 473 (Ser473) and threonine 308 (Thr308). The activated phosphoinositide-dependent kinase-1 and 2 (PDK-1, PDK-2) phosphorylate Akt at Thr308 and Ser473, respectively (10, 11). Activated, phosphorylated Akt (p-Akt) subsequently phosphorylates and activates various downstream proteins, including mammalian target of rapamycin (mTOR), and it is implicated in various cellular functions, including cell growth, proliferation and increased cell survival (12). Other mechanisms of activation of this pathway include loss of PTEN function by inactivating mutations, amplification or mutation of *PI3K* or *Akt*, activation by growth factor receptors and exposure to carcinogens (8). Activation of Akt by phosphorylation on Thr308 and Ser473 is one of the most common molecular alternations in human malignancies (13).

mTOR is an intracellular serine/threonine protein kinase of this signaling pathway, which plays a crucial role in carcinogenesis by regulating protein synthesis, cell growth and proliferation (14). Its functions are dependent on the fact that it forms complexes with other regulatory proteins leading to activation of at least two downstream translation proteins, eukaryotic translation initiation factor 4E binding protein (4EBP1) and S6 kinase 1 protein (S6K1) (15). These are regulators of protein translation for several other downstream signaling and transcription factors (16). Components of the PI3K/Akt/mTOR signaling pathway are frequently expressed in several human malignancies and these factors are considered targets for novel anticancer therapies (17). However, it is still unclear whether the evaluation of phosphorylated-mTOR (p-mTOR) expression is predictive of response to targeted therapy with mTOR inhibitors (18).

The mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in the regulation of normal cell differentiation by affecting cellular functions, such as the activity or localization of individual proteins, transcription of genes and increased cell cycle entry (19). The pathway activity is regulated by diverse extracellular signals and by products of several proto-oncogenes (20). Such stimulation leads to rapid activation by phosphorylation of members of the MAPK pathway, such as p42/p44MAPK proteins, which are serine/threonine kinases. A defect in the function of MAPK signaling pathway leads to uncontrolled cell growth and contributes to cancer development (21). The expression of phosphorylated MAPK (p-MAPK) is increased in actively proliferating tissues, such as normal proliferative-phase endometrium and endometrial carcinoma (22). In endometrial carcinoma, the MAPK signaling pathway appears to play a major role in tumor cell proliferation and survival (23). Recently, de-regulation of the MAPK pathway has been the subject of new anticancer targeted therapies (24).

Although the constitutive activation through phosphorylation of the PI3K/Akt/mTOR and MAPK signaling pathways are important in the development of various tumor types, only few studies have investigated these proteins in endometrial carcinomas (16, 17, 21, 24, 25, 27-42). Data regarding biomarkers of the PI3K/Akt/mTOR and MAP kinase pathways in endometrial carcinoma and their clinical significance are limited (31, 34). Previous reports have investigated immunohistochemical Akt expression in endometrial carcinoma (30, 31, 33, 35-41) and some have examined the correlation of Akt with clinicopathological parameters or outcome (30, 31, 33, 35, 37-39, 41). Similarly, immunohistochemistry (IHC) has been employed for the study of p-mTOR (16, 26, 28-30, 37, 39-42) and p-MAPK (25, 27, 33, 38) with evaluation of associations to clinicopathological parameters and outcome for mTOR (16, 26, 29, 30, 37, 39, 41, 42) and p-MAPK (27, 33, 38), respectively. Furthermore, the pI3K/Akt/m-TOR pathway is being therapeutically targeted in endometrial carcinoma and studies with PI3K, m-TOR, dual PI3K/m-TOR and Akt inhibitors are in progress (18).

The aim of the present study was to clarify the involvement of the PI3K/Akt/mTOR and MAPK signaling pathways in patients with type I endometrial carcinoma by analyzing immunohistochemically the expression of the activated proteins p-Akt, p-mTOR and p-MAPK, examine for correlations between them and with the significant clinicopathological factors, as well as with the outcome of endometrial carcinoma patients.

Patients and Methods

Patients and tissue samples. The medical records of patients treated for endometrial carcinoma in our institution between 1995 and 2010 were reviewed. Among the clinically identified cases, available tumor paraffin blocks were retrieved in 113 cases, which were subsequently studied. All patients had undergone surgical treatment with total abdominal hysterectomy and bilateral salpingo-oophorectomy. None of the patients received either neo-adjuvant chemotherapy or radiotherapy. All staging procedures

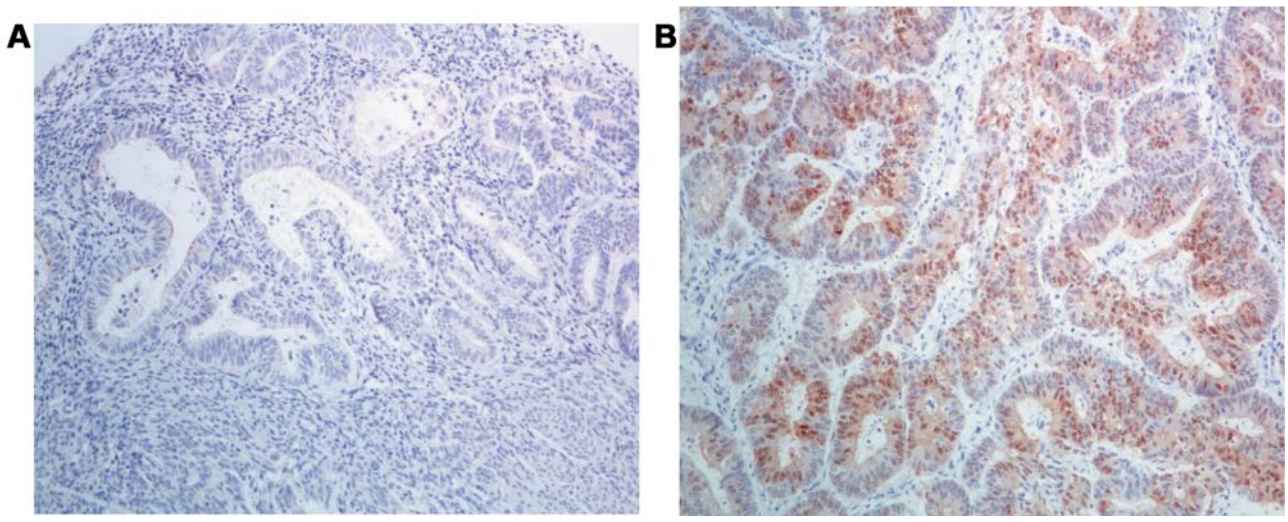

Figure 1. *Expression of p-Akt. (A) Negative p-Akt staining in non-neoplastic endometrium. (B) Moderate nuclear and cytoplasmic expression of p-Akt in endometrial carcinoma.*

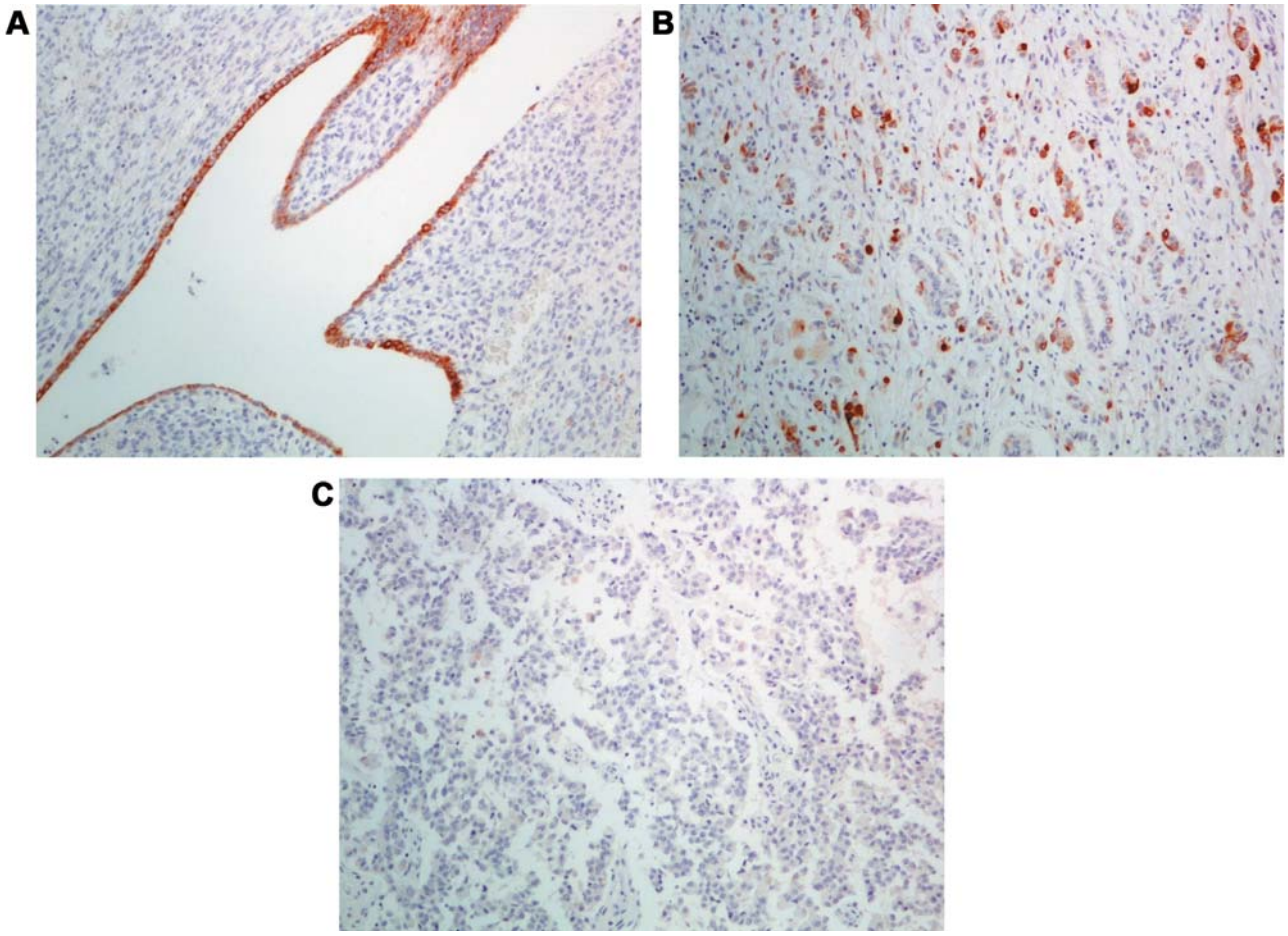

Figure 2. *Expression of p-mTOR. (A) Strong in non-neoplastic endometrium. (B) Scattered positive neoplastic cells. (C) The endometrial carcinomas were largely negative for expression of p-mTOR.*

Table I. *Primary antibodies, source and staining conditions.*

Antibody	Clone/Source	Dilution	Antigen retrieval
Phospho-p44/42 MAPK (Erk1/2) (thr202/Tyr204) rabbit mAb	20G11; CST	1:100	Citrate buffer pH 6.0
Phospho-Akt1/2/3 (Ser473) rabbit mAb	D9E XPTM;CST	1:50	Citrate buffer pH 6.0
Phospho-mTOR (Ser2448) rabbit mAb	49F9; CST	1:100	Citrate buffer pH 6.0

mAb, Monoclonal antibody; CST, Cell Signaling Technology Inc., Beverly, MA, USA.

Table II. *Patients' and tumors' characteristics of type I endometrial carcinoma.*

Characteristics		No. of patients (%)
Total number of patients		103
Patients' age	Median (years)	65±10.4
	Range	37-84
FIGO* stage	IA	43 (41.7%)
	IB	35 (34%)
	II	14 (13.6%)
	IIIA	6 (5.8%)
	IIIB	2 (1.9%)
	IIIC1	1 (0.8%)
	IVB	2 (1.9%)
Histological grade	Grade 1	40 (38.8%)
	Grade 2	45 (43.7%)
	Grade 3	18 (17.5%)
Myometrial invasion	<1/2	43 (41.7%)
	>1/2	60 (58.2%)
Cervical Invasion	Positive	24 (23.3%)
	Negative	79 (76.7%)
LVSI	Positive	31 (30.1%)
	Negative	65 (63.1%)
	No data	7 (6.7%)
Adjuvant therapy	Radiation	65 (63.1%)
	Chemotherapy	21 (20.4%)
OS (months)	Median (range)	41(1-180)
DFS (months)	Median (range)	38 (0-180)
Recurrence	No recurrence	63 (61.1%)
	Recurrence	18 (17.5%)
	No data	22 (21.4%)
Status	DOD	12 (11.7%)
	Alive	66 (64.1%)
	No data	25 (24.3%)

*FIGO, International Federation of Gynecology and Obstetrics stage (according to 2009 classification); DFS, disease-free survival; DOD, dead of disease; LVSI, lymphovascular space invasion; OS, overall survival.

were performed by gynecologists and further treatment options were discussed and decided upon by the gynecologic oncology tumor board of our institution.

The specimens were examined in the Department of Pathology of our Institution. All tissue slides for every case were reviewed by one pathologist (HPK) for assessment of the tumor type, histological grade, depth of myometrial invasion, invasion of the cervix, presence of lymphovascular space invasion (LVSI) and presence of pelvic or extrapelvic disease extension. The histological classification was performed using the WHO criteria (34). From 113 carcinomas, 103 were type I (91%) and 10 were type II carcinomas (9%). The latter were not analyzed statistically. Surgical staging reported in the study was performed according to the 2009 staging system for endometrial carcinoma established by the International Federation of Gynecology and Obstetrics (FIGO) (35).

The patients were evaluated regularly, every 6 months, for the first two years and every year for the following years for disease recurrence by clinical examination and diagnostic imaging methods, such as computed tomography (CT) scan and magnetic resonance imaging (MRI). Measurement of tumor markers was also performed on regular basis every 6 months for the first two years. This study was approved by the Ethical committee of the University Hospital of Patras School of Medicine.

Immunohistochemical assay. For each case, a representative formalin-fixed, paraffin-embedded (FFPE) tissue block was selected containing, preferentially, non-neoplastic endometrial tissue adjacent to the tumor. Non-neoplastic endometrium was present adjacent to the tumor in 61 cases. Serial 3 μm tissue sections were cut, mounted on adhesive slides and subjected to immunohistochemical labeling. Briefly, the slides were de-paraffinized in xylene, rehydrated in a series of graded ethanol solutions, incubated in 0.3% H_2O_2 for 15 min at room temperature to block endogenous peroxidase activity and submitted to antigen retrieval by microwave heating in 10 mM citrate buffer, pH 6.0 for 15 min. After cooling at room temperature, the slides were incubated with 1% bovine serum albumin for 30 min at room temperature and, then, incubated with the primary antibodies for 1 h at room temperature. The primary antibodies, source and staining conditions are shown in Table I. Dako EnVision labeled polymer (Dako, Carpinteria, CA, USA) was used as detection system, DAB (Dako) as chromogen and Harris hematoxylin was applied for nuclear counterstaining. Sections from breast carcinoma were used as positive controls.

Evaluation of immunohistochemical staining. The immunoreactivity was assessed by one pathologist (HPK), blinded as to the clinical characteristics of the tumors and the patients' outcome data. The entire tissue present on the examined section was evaluated. The neoplastic and non-neoplastic tissues were evaluated separately. The intensity and the percentage of positively stained cells were recorded. The localization of the staining reaction, whether nuclear, cytoplasmic or both nuclear and cytoplasmic, was also noted. The immunoreactivity was expressed by calculating the H-score as follows: H-score=(1×percentage of weakly positive cells)+(2×percentage of moderately strong positive cells)+(3 × percentage of strongly positive cells), which would range from 0 to 300.

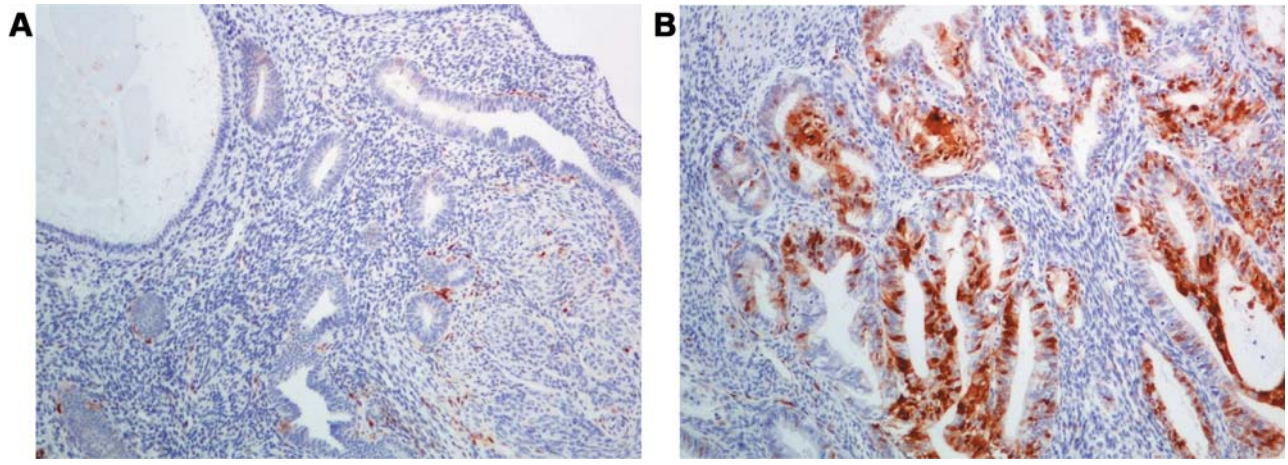

Figure 3. *Expression of p-MAPK. (A) Negative p-MAPK in non-neoplastic endometrium (B). Strong expression of p-MAPK in endometrial carcinoma.*

Statistical Analysis. Statistical analysis was performed using a software package (SPSS for Windows 16.0; SPSS Inc., Chicago, IL, USA). The expression of the examined factors was examined for association with clinicopathological parameters and outcome, namely overall survival (OS) and disease-free survival (DFS). The distribution of H-scores did not follow a normal distribution according to Kolmogorov-Smirnov test and, thus, non-parametric tests were used for statistical analysis. Values were treated in a continuous scale for all tests except from survival analysis with Kaplan Meier curves, where the median value for each marker was used as a cut-off to dichotomize cases into low and high expression. The Wilcoxon test was used for comparisons between paired samples of non-neoplastic and tumor tissue. The Kruskal Wallis test was used for comparisons between groups. Correlations between the expression of the markers was examined by the Pearson correlation test. Follow-up was available for 76 patients. Survival analysis was performed with Kaplan Meier curves and differences between groups were calculated with the log-rank test. Multivariate analysis was performed with Cox's regression analysis.

Results

Clinical and histopathological data. The patients' and tumors' characteristics are summarized in Table II. One hundred and three cases were included in the study. The patients were 37-84 years old (mean age=64 years, median=65 years). Among type I carcinomas, 40 (38.8 %), 45 (43.7%) and 18 (17.5%) were grade 1, 2 and 3, respectively. Forty three (41.7 %) tumors were stage IA, 35 (34%) were stage IB, 14 (13.6 %) were stage II, 9 (8.7 %) were stage III and 2 (1.9 %) were stage IV. LVSI was present in 31 (30.1%) cases, absent in 65 (63.1%) cases and not evaluable in 7 (6.8%) cases because not all the slides were available for review. Adjuvant radiotherapy and adjuvant chemotherapy were administered in 65 (63.1%) and 21 (20.4%) patients, respectively. Eight (7.6%) patients received no additional treatment, whereas for 9 (8.7%) patients no data were available in our medical records as to the administration of adjuvant treatment. The DFS was 0-180 months (mean=47.9, median=38.0) and the OS was 1-180 months (mean=52.9, median=41). During the follow-up period, 12 patients died of disease, 4 patients were alive with disease, 62 patients were alive showing no evidence of the disease and 25 (24.3%) patients were lost to follow-up.

Expression of p-Akt in type I endometrial carcinoma. The expression of p-Akt was observed only in the neoplastic epithelial cells, in a patchy pattern or in scattered tumor cells and was not observed in the endometrial stromal cells (Figure 1). In 31 (30.7 %) cases, p-Akt staining was nuclear only, usually of weak-to-moderate intensity, in 15 (14.9 %) cases was both cytoplasmic and nuclear and in 1 (1%) case p-Akt staining was only cytoplasmic. Among the carcinomas, 54 (53.5%) were negative for p-Akt. The H-score for p-Akt ranged from 0-70 (mean=5.3±12.1) in the neoplastic tissue (Table III). The non-neoplastic endometrium was negative for p-Akt in all examined cases.

Expression of p-mTOR in type I endometrial carcinoma. The expression of p-mTOR was also observed in a patchy pattern, both in tumors and non-neoplastic endometrium (Figure 2). In tumors, p-mTOR expression was negative in 18 cases (17.4%). Cytoplasmic and nuclear or only nuclear positivity was observed in 9 (8.7%) and 73 (72.3%) cases, respectively (Table III). In the non-neoplastic tissue, p-mTOR expression was negative in 1 (2.1%) case, cytoplasmic in 43 (91.5%) cases, cytoplasmic and nuclear in 1 (2.1%) case and nuclear only in 2 (4.3%) of the cases (Table IV). The H-score for p-mTOR ranged from 0-130 (mean=22±28) for tumors and from 0-210 (mean=53±60) for non-neoplastic tissue (Tables III, IV). The p-mTOR

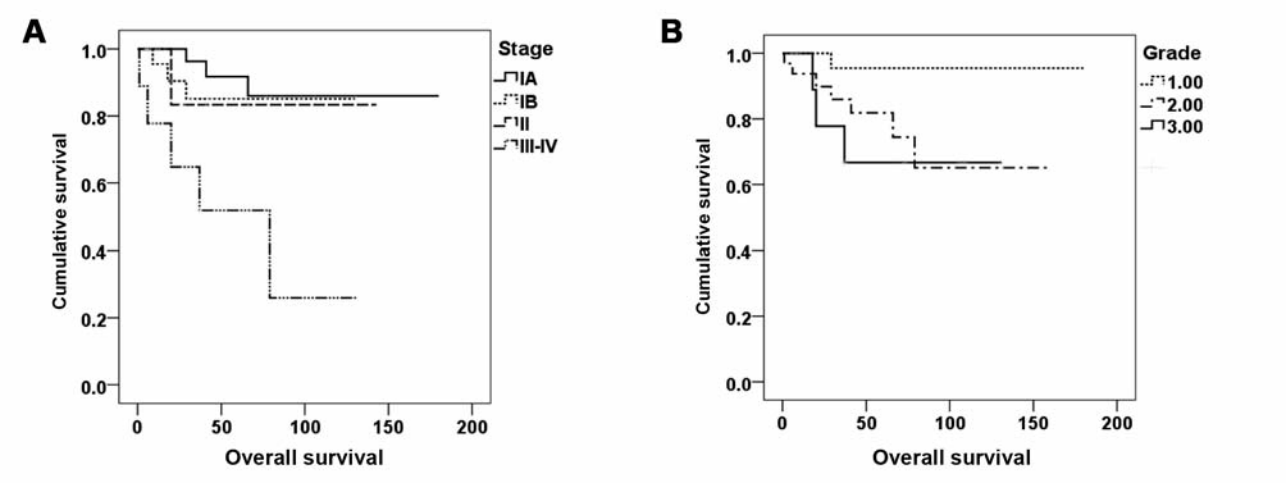

Figure 4. *Kaplan Meier curves for overall survival (months) calculated by (A) stage, p=0.002 and (B) grade, p=0.069.*

expression was higher in non-neoplastic endometrium than in carcinomas ($p<0.001$).

Expression of p-MAPK in type I endometrial carcinoma. The expression of p-MAPK was also patchy, observed in tumors and non-neoplastic endometrium, as well as in endothelial cells, scattered myometrial fibers and endometrial stromal cells (Figure 3). Seventeen carcinomas (16.8%) were negative for p-MAPK expression. In 73 (72.3%) cases, p-MAPK expression was usually both in the nucleus and the cytoplasm of the neoplastic cells, in the nucleus only in 10 cases (9.9%) and in 1 case (1%) it was restricted to the cytoplasm (Table III). Among the 61 cases with non-neoplastic tissue present adjacent to the tumor, 31 (50.8%) cases were negative for p-MAPK, 21 (34%) and displayed both nuclear and cytoplasmic staining, 8 (13.1%) nuclear only and 1 (1.6%) case showed only cytoplasmic expression (Table IV). The nuclear staining was usually observed in tumors with low H-scores ranging from 1-10. The H-score for p-MAPK ranged from 0-180 (mean=29±45) for tumors and from 0-140 (mean=9±21) for the non-neoplastic tissue (Table III, IV). p-MAPK expression was higher in tumors than in non-neoplastic tissue ($p=0.001$).

Association of examined proteins with clinicopathological parameters and outcome. Expression of p-Akt was correlated with p-MAPK (r=0.229, $p=0.022$) and with p-mTOR (r=0.218, $p=0.029$). The expression of the examined markers was not associated with OS, DFS, stage, grade, LVSI, adjuvant radiation therapy, chemotherapy, recurrence of disease and survival, except for a marginal correlation of p-Akt with LVSI ($p=0.055$). OS was associated with LVSI ($p=0.002$), stage ($p=0.002$), chemotherapy (CMT) ($p=0.007$) and marginally with grade ($p=0.069$) in univariate analysis (Figure 4). DFS was associated with LVSI ($p<0.001$), stage ($p<0.001$), grade ($p<0.001$), CMT ($p<0.001$) in univariate analysis (Figure 5). Multivariate analysis that included tumor grade and stage, patient's age, presence of LVSI and treatment with radiotherapy (RT) and/or CMT showed that stage IB *versus* III/IV ($p=0.043$, ExpB=0.102, 95% confidence interval (CI)=0.011-0.926) and treatment with chemotherapy ($p=0.011$, ExpB=0.183, 95%CI=0.049-0.683) were independently associated with DFS. Stage IB and II *versus* III/IV ($p=0.016$, ExpB=0.442, 95%CI=0.003-0.558 and $p=0.012$, ExpB=0.040, 95%CI=0.003-0.497, respectively) were independently associated with OS.

Discussion

In this study, the expression of p-Akt was noted in the minority of endometrial carcinomas and in low percentage of the neoplastic cells, while it was not seen in non-neoplastic endometrium. Our findings are in contrast with those of previous studies, that have showed significant staining for p-Akt in endometrial carcinomas (30-31, 35, 37, 39). The divergence among various studies of the levels of p-Akt expression may be attributed to the instability of phosphoepitopes of Akt (10). Furthermore, antibodies for p-Akt are known to present significant difficulties (45). Despite the difference in the level of p-Akt expression, our results are in agreement with previous studies demonstrating increased expression of p-Akt in endometrial carcinoma compared to non-neoplastic endometrium (31), implying activation of the Akt pathway in this tumor type. Phosphorylated Akt detaches from the cell membrane and, subsequently, phosphorylates substrates both in the cytoplasm and the nucleus (10), while it has been identified in both cellular compartments by immunohistochemistry.

The significance of nuclear or cytoplasmic Akt localization has not been fully elucidated. Nuclear localization of p-Akt may result in phosphorylation and inactivation of p21 and p27 cyclin-dependent kinase inhibitors, thus resulting in cell cycle progression (46). In a recent study, the nuclear p-Akt labeling index was higher in endometrial carcinoma compared to normal endometrium (31). On the other hand, some reports suggest that cytoplasmic Akt has oncogenic function (47, 48). In pancreatic cancer, the cytoplasmic expression of p-Akt was associated with poor prognosis, whereas high nuclear staining had a favorable prognosis (49). A significant association was also found between cytoplasmic expression of p-Akt and reduced DFS in breast cancer (50). Mori and colleagues reported mostly cytoplasmic p-Akt staining in endometrial carcinoma, although they observed both cytoplasmic and nuclear staining in certain cases. Nonetheless, they did not observe any correlation of Akt staining with prognosis or clinicopathological parameters (37). In the present study, the expression of p-Akt was more often nuclear, while both nuclear and cytoplasmic localization was noted in fewer cases.

Although p-Akt expression has been associated with aggressive behavior in several tumor types (10), data on endometrial carcinoma are limited. Abe and coworkers have associated nuclear p-Akt with poor prognosis in grade 1 endometrial carcinoma (31). Similarly, in a recent study, higher expression of p-AKT in endometrioid endometrial adenocarcinoma was associated with positive lymph nodes and poor progression-free survival (PFS) and OS (33). In the present study, we were not able to demonstrate a prognostic role for the localization of p-Akt expression in endometrial carcinoma or correlation between p-Akt expression and clinicopathological parameters, in agreement with most previous reports (30, 35, 37-39, 41). These findings suggest that p-Akt expression is unlikely to convey prognostic information in endometrioid endometrial carcinoma.

In the present series, p-AKT expression was significantly associated with the expression of p-mTOR, unlike a previous report (37). In addition, p-AKT expression was also significantly associated with the expression of p-MAPK, thus providing evidence to support the crosstalk between the Akt-mTOR and MAPK pathways. In the HepG2 cell line, p-Akt levels have been shown to be modulated by estradiol through the modification of PTEN levels by the ERK pathway, linking the Akt-mTOR and MAPK pathways (51). Accumulated data, reviewed by Mendoza and coworkers, indicate both positive and negative crosstalk mechanisms between the PI3K/Akt/mTOR and MAPK pathways, varying between different cancer types (52). Our data suggest a positive relation between these pathways in endometrial carcinoma, similar to previous reports (37). Unlike our results, other investigators did not observe any association between the Akt and MAPK pathways (38).

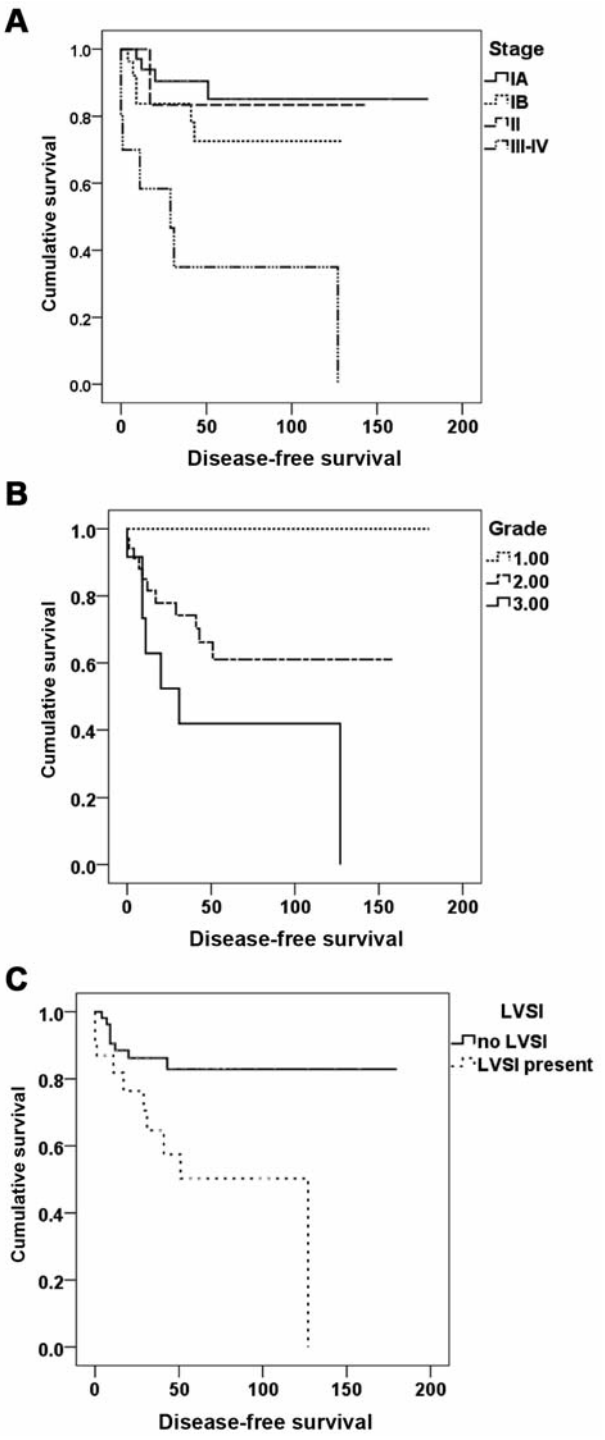

Figure 5. *Kaplan Meier curves for disease-free survival (months) calculated by (A) stage of disease, p<0.001 (B) grade, p<0.001 and (C) lymphovascular space invasion (LVSI), p=0.005.*

Expression of p-mTOR was observed in the vast majority of tumors, localized largely in the nuclei. The expression was patchy, a finding that has also been described by previous

investigators (16). Expression of p-mTOR was even higher in the non-neoplastic endometrium compared to the tumor, a difference of statistical significance; albeit in benign endometrium the expression of p-mTOR was noted in the cytoplasm of the endometrial epithelium. Expression of p-mTOR in both endometrial carcinoma and benign endometrium was also observed in the study of Wahl and colleagues (40). The significance of the localization of p-mTOR expression is still a matter of debate. mTOR is localized predominantly in the cytoplasm and directly or indirectly critically involved in complex signaling networks regulating many cellular events, such as the initiation of translation (15). Nonetheless, reports indicate that mTOR may shuttle between nucleus and cytoplasm and a small fraction of mTOR is found in the nucleus in a steady state both in normal and malignant cells (53-55). Furthermore, it has been reported that the mTOR cytoplasmic-nuclear shuttling appears to be required for cytoplasmic functions of mTOR, such as anti-apoptosis (56, 57). Recent data in endometrial tumorigenesis postulated that nuclear localization of mTOR may participate in activation of nuclear 4E-BP1 through phosphorylation and contributes to tumor progression by regulating the cell cycle progression and apoptosis (16). Yoshida and coworkers assigned particular importance in the nuclear localization of p-mTOR in endometrial carcinoma concluding that it is critical for tumor progression and associated only nuclear p-mTOR with shorter recurrence-free survival (RFS) (30). Choi and colleagues described an association of higher cytoplasmic p-mTOR with better survival in univariate analysis (29), but Vandenput *et al*. found an association of cytoplasmic p-mTOR in recurrent endometrial carcinomas with worse survival (42). Localization of p-mTOR has also been assessed in breast (58) and gastric (59) carcinoma where cytoplasmic expression was associated with worse prognosis in both tumor types, while nuclear p-mTOR was associated with better RFS and OS in gastric carcinoma (59). Irrespective of the clinical associations, our findings of primarily nuclear localization of both p-Akt and p-mTOR in the neoplastic cells suggest, in contrast to the cytoplasmic localization of p-mTOR in non-neoplastic endometrium, a nuclear mode of action of the Akt/mTOR pathway in endometrial carcinogenesis. Further investigation is required to identify the exact nuclear function of this protein and the prognostic role of its localization in the cellular compartments of endometrial carcinoma. Our results suggest that evaluation of nuclear localization of p-mTOR may prove useful, in future studies assessing the predictive value of p-mTOR for targeted therapies. Furthermore, the patchy character of the expression for both p-Akt and p-mTOR would imply that the evaluation of these markers may be more reliable in whole-tissue sections rather than in tissue microarrays (TMAs).

Table III. *Immunohistochemical expression of p-Akt, p-mTOR and p-MAPK in type I endometrial carcinomas.*

Expression by IHC, localization	p-Akt expression	p-mTOR expression (%)	p-MAPK expression (%)
Negative	54 (53.5)	18 (18)	17 (16.8)
Positive, Cytoplasmic only	1 (1)	0 (0)	1 (1)
Positive, Nuclear only	31 (30.7)	73 (73)	10 (9.7)
Positive, Cytoplasmic and nuclear	15 (14.9)	9 (9)	73 (70.9)
N	101	100	101
Mean H-score	5.34	21.91	29.04
Median H-score	<1	10.00	10.00
SD	12.1	27.8	44.6
Minimum	0.00	0.0	0.0
Maximum	70.0	130.0	180.0

IHC, Immunohistochemistry; N, number; SD, standard deviation.

Regardless of the localization, the prognostic significance of p-mTOR expression in endometrial cancer has been controversial. Certain reports demonstrated increased cytoplasmic p-mTOR in high grade and stage (T2-T4) endometrial tumors with lymph node metastases (16, 30) or with worse survival in recurrent tumors (42). Others demonstrated an association between increased expression of cytoplasmic p-mTOR and better survival (29). Most investigators, however, failed to find a correlation of p-mTOR expression with clinicopathological parameters or outcome in endometrial carcinoma (26, 37, 39, 41). In agreement with these reports we did not identify any correlation between expression of p-mTOR and clinicopathological features or survival.

Expression of p-MAPK in normal and malignant endometrial tissues was, in agreement with previous reports, also patchy in distribution and, most commonly, both nuclear and cytoplasmic (22, 27, 31, 38, 60). Expression of p-MAPK was observed in the vast majority of endometrial carcinomas with increased expression in endometrial carcinoma compared to non-neoplastic endometrium, suggesting that the MAPK pathway contributes to endometrial carcinogenesis. Our results are in agreement with the findings of previous studies (22, 27, 33). Activation of MAPK pathway has also been implicated in the development of other steroid-dependent tumors. In breast cancer, increased levels of p-MAPK were detected in malignant breast tissue compared with the surrounding benign breast tissue (61, 62). Contrary to these observations, the study of Abe *et al*. described similar levels of p-MAPK expression among normal, hyperplastic and neoplastic endometrial tissues (31) and Desouki *et al*., by western blot analysis, noted that elevated

Table IV. *Immunohistochemical expression of p-mTOR and p-MAPK in non-neoplastic tissue.*

Expression by IHC, localization	p-mTOR expression (%)	p-MAPK expression (%)
Negative	1 (2.1%)	31 (50.8%)
Positive, Cytoplasmic only	43 (91.5%)	1 (1.6%)
Positive, Nuclear only	2 (4.3%)	8 (13.1%)
Positive, Cytoplasmic and nuclear	1 (2.1%)	21 (34.4%)
N	59	61
Mean H-score	53.6	9.1
Median H-score	20.0	0.0
SD	60.3	21.1
Minimum	0.0	0.0
Maximum	210.0	140.0

IHC, Immunohistochemistry; N, number; SD, standard deviation.

expression of p-MAPK was detected in benign endometrium rather in endometrial carcinomas (25). These investigators suggested that progression from normal to malignant endometrium is independent of p-MAPK expression.

The prognostic role of p-MAPK expression in endometrial carcinoma is still unclear. In the present study, p-MAPK was not correlated with survival. Our results are in agreement with previous reports in endometrial carcinoma describing the absence of prognostic function of MAPK in endometrial carcinoma (33, 38). We also found no association with clinicopathological parameters, similarly to previous studies (27, 31, 38). Contrary to these observations, Mizumoto *et al*. noted that low levels of p-MAPK expression were associated with significantly lower RFS and OS (27), Zhou *et al*. found that expression of p-MAPK in endometrial carcinoma was significantly associated with FIGO stage (60) and Castellvi *et al*. noted that cytoplasmic p-MAPK expression was associated with higher stage, grade and deep myometrial invasion (38). In pancreatic cancer, high expression of p-MAPK was correlated with shorter survival (63) but, in breast cancer, p-MAPK expression was not associated with clinical outcome (64). The above findings indicate that, in the future, large prospective studies are necessary to clarify the relationship between the activation of MAPK pathway in endometrial cancer and patient prognosis.

Conclusion

Although we did not identify any prognostic value for p-Akt, p-mTOR and p-MAPK in type I endometrial carcinoma, the significantly increased levels of p-Akt and, in particular, p-MAPK in carcinoma compared to benign endometrium support the involvement of these pathways in endometrial tumorigenesis and their potential for therapeutic targeting. Our observation of the patchy and heterogeneous distribution of the staining for p-Akt and p-MAPK in the tissues would suggest preferential investigation of these markers in whole-tissue sections rather than in TMAs in future studies.

The better understanding of these signaling pathways and the identification of biomarkers would be crucial for the development of targeted-therapies in endometrial cancer. Since predictive markers for these pathways have not yet been identified and the predictive function of p-Akt, p-MAPK and p-mTOR has not yet been adequately evaluated, further investigation of these markers in large prospective studies might provide insight to the possible interactions between the pathways and clarify their prognostic or predictive value.

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