RESEARCH ARTICLE

Prevalence and Clinical Significance of Mammalian Target of Rapamycin Phosphorylation (p-mTOR) and Vascular Endothelial Growth Factor (VEGF) in Clear Cell Carcinoma of the Ovary

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Abstract

Background: To determine the prevalence of mammalian target of rapamycin phosphorylation (p-mTOR) and vascular endothelial growth factor (VEGF) and any correlation with clinical characteristics and prognosis in ovarian clear cell carcinoma patients. Materials and Method: Seventy four paraffin-embedded specimens of such carcinomas frompatients who underwent surgery, received adjuvant chemotherapy and were followed up at King Chulalongkorn Memorial Hospital during January 2002 to December 2008 were stained with rabbit monoclonal IgG p-mTOR and rabbit polyclonal IgG VEGF using immunohistochemical methods. Medical records were reviewed and clinical variables were analysed. Results: The prevalence of positive p-mTOR in ovarian clear cell carcinoma was 87.9% and significantly higher in advance-stage than early-stage patients (100% versus 83.6%, P<0.05). Two-year disease free survival and 2-year overall survival in patients with positive p-mTOR expression were 60% and 69.2% with no differences from patients with negative p-mTOR expression (p>0.05). The prevalence of VEGF expression was 63.5% and significantly higher in chemo-sensitive than chemo-resistant patients (70.7% versus 37.5%, P<0.05). Two-year disease free survival and 2-year overall survival in patients with VEGF expression were 72.3% and 83% respectively which were significantly different from patients with negative VEGF expression (p<0.05). Conclusions: p-mTOR expression in ovarian clear cell carcinoma was significantly correlated with the stage of disease. VEGF expression was significantly correlated with chemosensitivity, and survival. Further studies of related targeted therapy might be promising.

Keywords: p-mTOR - VEGF - ovarian clear cell carcinoma - immunohistochemistry

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Introduction

Ovarian cancer has the highest mortality rate among gynecologic malignancies worldwide. The incidence rate was 9.8 per 100,000 woman-year and mortality rate was 4.9 per 100,000 woman-year (Jemal et al., 2009). It is also the sixth most frequent cause of cancer death in Thai women. The most common histologic type is epithelial stromal ovarian cancer, which is approximately 90% of all ovarian malignancies. The incidence of clear cell carcinoma is reported to be 4-6% of all histologic subtypes among epithelial stromal ovarian cancer throughout the world (Jemal et al., 2009). There have been three major clinical problems in management of ovarian clear cell carcinoma. First is its poor response to standard platinum-based chemotherapy regimens; second is the association with a worse prognosis than the more common serous adenocarcinoma of ovary and lastly, advanced cases have short survival periods. Although chemoresistance is multifactorial, it is well accepted that resistance to programmed cell death is a major contributing cause. There are two forms which have been described: 1) apoptosis or caspase-dependent cell death. 2) caspase-independent cell death

The most characterized caspase independent programmed cell death pathway is autophagy, involving the controlled formation of autophagosomes. The autophagosome is a double-membrane cytoplasmic vesicle, which can fuse with lysosomes, resulting in the digestion of molecules within the autophagosome. It is controlled by the Akt-mammalian target of rapamycin (mTOR) pathway.

Mammalian Target of Rapamycin (mTOR), a large 289 kDa serine/threonine protein kinase complex, is well

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conserved in many eukaryotes including mammals and yeast and is also a prime strategic target for therapeutic research against cancer. The mTOR pathway is emerging as an integrator of many signals that control cell growth and proliferation. mTOR is also a critical component of the phosphatidylinositol 3- kinase (PI3K) and protein kinase B (Akt) pathways, effecting signals that activate downstream protein kinase required for both ribosomal biosynthesis and translation of mRNAs of proteins. These proteins are essential for G1 to S phase traverse (Noske et al., 2008; Miyazawa et al., 2009; Trinh et al., 2009; Zhang et al.,2009). The functions of mTOR are a central controller of protein translation and cell cycle progression. The complex regulates cell growth and metabolism by modulating processes such as protein synthesis, ribosome biogenesis, autophagy and angiogenesis inhibition. A previous study (Trinh et al., 2009), found the activated substrate, mTOR phosphorylation (p-mTOR), was greater in clear cell carcinoma of ovary.

Tumor growth and the metastatic process are dependent on the formation of new blood vessel. A well-developed vascular network enhances metastasis, which depends on the production of angiogenic factors (VEGF) by host and tumor cells. Angiogenesis may be a prognostic factor in various solid tumors such as breast, prostate, gastric and non-small cell lung cancers (Huynh et al., 2007; Miyazawa et al., 2009; Trinh et al., 2009; Yap et al., 2009). However, results in ovarian cancer are conflicting and its clinicopathologic significance is still uncertain (Hidalgo et al., 2000; Huang et al., 2003; Mita et al., 2003; Altomare et al., 2004; Chan et al., 2004; Meric-Bernstam et al., 2004; Rowinsky et al., 2004; Sönmezer et al., 2004; Janus et al., 2005; Springett et al., 2005; Xing et al., 2005; Chon et al., 2006; Smolewski et al., 2006; Yagyu et al., 2006; Huynh et al., 2007; Noske et al., 2008; Mabuchi et al., 2009; Miyazawa et al., 2009; Trinh et al., 2009; Yap et al., 2009; Zhang et al., 2009). VEGF also plays an important role in malignant ascites formation, angiogenesis, and ovarian tumor growth; whereas mTOR pathway regulates VEGF expression in cancer cells through mTOR-HIF-1α-VEGF pathway. It may involve the inhibition of angiogenesis.

The aims of this study were to determine the prevalence of expression of phospoho- mTOR (p-mTOR) and VEGF and to examine their correlation with stage of disease, chemosensitivity and prognostic significance in clear cell carcinoma of ovary.

Materials and Methods

Patients

The study sample was seventy-four ovarian clear cell carcinoma patients , who underwent surgical staging or tumor biopsy and received standard adjuvant chemotherapy and were followed at King Chulalongkorn Memorial Hospital (KCMH) between January 2002 and December 2008. Paraffin blocks of the tumor specimens and medical records were available in all cases. This study was approved by Chulalongkorn University Ethical Committee. Clinicopathologic stage was determined according to the 2009 FIGO staging system classification. Criteria of chemo-sensitive patient was no tumor recurrence or recurrence after 6 months. Chemo-resistant patient recurred within 6 months of chemotherapy cessation. Early-stage included stage I or II and advance-stage included stage III or IV according to the 2009 FIGO staging system classification. The patient and tumor characteristics are summarized in Table 1. Sample size was calculated by α=0.05 and 10% acceptable error.

Study design retrospective descriptive study

Sample size:

Np-mTOR = $[Z^2\alpha/_2\ p\ (1-p)]/d2$

$Z\alpha/2 = 1.96 \quad (\alpha = 0.05)$

- p-mTOR in Clear cell carcinoma of ovary = 86.4%[8]
  p = 0.86
  d = acceptable error = 10% of p = 0.08
  N = 70

$N_{VEGF} = [Z^2\alpha/_2\ p\ (1-p)]/d2$

$Z\alpha/2 = 1.96 \quad (\alpha = 0.05)$

- VEGF in Clear cell carcinoma of ovary = 88%[4]
  p = 0.88
  d = acceptable error = 10% of p = 0.08
  N = 63

Immunohistochemistry

Formalin-fixed, paraffin-embedded 4-μm sections were stained using standard immunohistochemical methods. Each section was deparaffinized and antigen was retrieved using sodium citrate, pH 6.0 and boiled in microwave oven. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. Sections were incubated with 3% normal horse serum for 20 min and then stained with primary anti-phospho-mTOR (Ser2448) (49F9), rabbit monoclonal IgG antibody (IHC Specific) (Cell Signaling, USA), dilution 1:100 and anti-VEGF (A-20) (SC-152), rabbit polyclonal IgG antibody (Santa Cruz, USA), dilution 1:100 for 1 h. UltraView Universal DAB detection kit as secondary antibody was incubated with the samples for 30 min. Diaminobenzene was used as chromogen and Meyer's hematoxylin was used as counterstain. Negative control was the section which was performed without primary antibody and a section of bladder transitional epithelial carcinoma, breast carcinoma and colon carcinoma (for p-mTOR). A section of breast

Table 1. p-mTOR and VEGF Expression According to Clinical Characteristics

	Positive p-mTOR n(%)	p	Positive VEGF n(%)	p
N (%)	65.00 (87.9%)		47.00 (63.5%)	
Mean (%)	52.80		51.90	
Early stages* 55 (74.3%)	46.00 (83.6%)		38.00 (69.1%)	
Mean	50.09		55.90	
Advance stages* 19 (25.7%)	19.00 (100%)	0.04	9.00 (47.4%)	0.27
Mean	60.73		40.57	
Chemo-sensitive pt.** 58 (78.3%)	51.00 (87.9%)		41.00 (70.7%)	
Mean	54.36		57.79	
Chemo-resistant pt.*** 16 (21.7%)	14.00 (87.6%)	0.63	6.00 (37.5%)	0.01
Mean	47.25		26.12	
No Endometriosis 45 (60.8%)	40.00 (88.9%)	0.73	28.00 (62.2%)	0.65
Endometriosis 29 (39.2%)	25.00 (86.2%)		19.00 (65.5%)	

*Early stage included stage I or II, advance stage included stage III or IV according to the 2009 FIGO staging system classification. **No tumor recurrence or recurrence after 6 months. ***Tumor recurrence within 6 months

carcinoma and melanoma (for VEGF) were used as a positive control in this study. All the staining was done on the BenchMark® XT (Ventana Medical System Inc.) autostainer (product number N750-BMKXT-FS).

Outcome evaluation

A gynecologic pathologist blinded to the clinical data reviewed all H&E slides and selected the appropriate sections for immunohistochemical studies. Three regions of greatest immunostaining (hot spots) were selected and 100 cells in each area were counted for the percentage of immunoreactive cells as standard protocol. If the percentage of immunoreactive cells was more than 10%, the sample was classified as positive p-mTOR and VEGF expression (Miyazawa et al., 2009; Trinh et al., 2009). Expression was semiquantitatively assessed according to the intensity of the cytoplasmic staining (no staining = 0, weak staining = +1, moderate staining = +2, strong staining = +3). The distribution staining extent in the tumor cells was classified as follows: (0–10% = 0; 11–50% = 1+; 51–75% = 2+; 76–100% = 3+). The slides were examined under a bright field microscope. Tumors with cytoplasmic staining of +2 or +3 were subgrouped as the strong-staining group, staining of +1 were subgrouped as a the weak-staining group and tumors with staining extent 1+(11-50%) were subgrouped as low grade group, whereas tumors with staining extent 2+ (51-75%) or 3+ (76-100%) were subgrouped as high grade group.

Statistical analysis

The quantitative results are presented as mean±SD. The correlation between p-mTOR and VEGF expression was using bivariate correlation and the associations between p-mTOR and VEGF expression and clinicopathologic variables were analyzed by Chi-square test. Survival analysis was generated by Kaplan– Meier method and Log rank test was used to assess the statistical significance. A p-value less than 0.05 was considered statistically significant(SPSS version 17.0 for Windows; SPSS, Chicago, IL, USA).

Results

Thirty-eight of all 74 cases (51.4%) were postmenopausal woman and mean age of all patients in study was 53±9.9 years. The mean preoperative CA125 level was 518.1 U/ml. Most of ovarian clear cell carcinoma patients were in stage I, had undergone optimal surgery, were sensitive to first line adjuvant chemotherapy and had no endometriosis association. Sixty-five (87.9%) of the patients showed positive p-mTOR expression (mean 52.8±26.9%), mainly of them were high grade (56.8%) and strong intensity (62.2%). The p-mTOR was significantly expressed in advanced stage (100%) [mean 60.73% ($p<0.05$)]. Positive VEGF expression was detected in 47/74 (63.5%) (mean 51.9±41.6%) and mainly high grade (58.1%) with strong intensity (39.2%) (Table 1). The VEGF was expressed in early stage (69.1%) but had no statistically significant difference ($p>0.05$): from advanced stage, VEGF was significantly expressed in 41 (70.7%) in chemo-sensitive patients [mean 57.79% ($p<0.05$)]. The p-mTOR expression in chemosensitive and chemoresistant groups were similar [87.9% vs 87.6%($p>0.05$)]. There was no significant difference between p-mTOR and VEGF expression and the presence or absence of endometriosis [table 1]. No significant correlation was demonstrated between p-mTOR and VEGF expression (r = -0.002) (Figure 1).

The median time of follow up was 60 months (range 44-75). During this period, 38 patients died of recurrent disease (51.4%). Cancer recurrence was detected in 44 patients (59.5%) and the median time to recurrence was 34 months (range 16-51). Twenty-six in 44 recurrent patients (59%) were chemo-sensitive groups whereas chemo-resistant groups were detected in 18/44 (40.9%) (Table 2). Positive VEGF expression group was associated with significantly longer overall survival (OS) than negative group (Figure 3) but not with disease free survival (DFs) (Figure 2). There was no significant difference between presence and absence of p-mTOR expression in OS and DFs (Figure 2 and 3). Two-year DFs and 2-year OS in ovarian clear cell carcinoma patients were 59.5% and

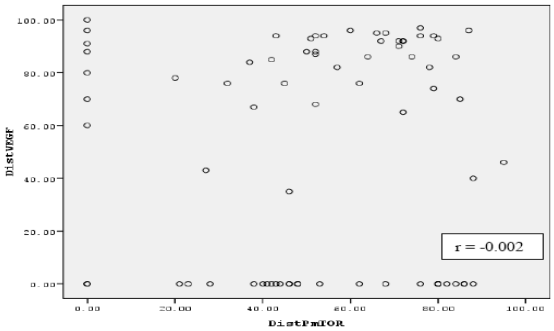

Figure 1. Correlation between p-mTOR and VEGF Expression

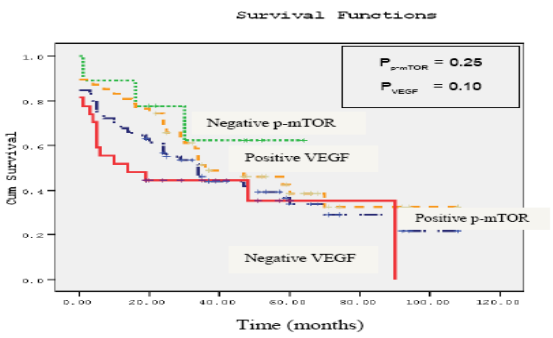

Figure 2. Disease Free Survival According to p-mTOR and VEGF Expression

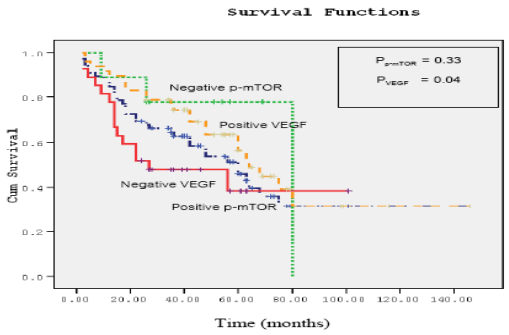

Figure 3. Overall Survival According to p-mTOR and VEGF Expression

Table 2. Disease Free Survival and Overall Survival

Dead n (%)	38 / 74 (51.4%)
Recurrence n (%)	44 / 74 (59.5%)
Median disease free survival, months (range)	34 [16-51]
Median overall survival, months (range)	60 [44-75]
2-year disease free survival n (%)	44 / 74 (59.5%)
2-year overall survival n (%)	53 / 74 (71.6%)

Table 3. Correlation between p-mTOR and VEGF Expression and 2-year Disease Free Survival and 2-year Overall Survival

		2yr DFs	p	2yr OS	p
P-mTOR	Positive (65)	60%	0.800	69.20%	0.220
	Negative (9)	55.60%		88.90%	
	Low grade	53.10%		68.80%	
	High grade	64.30%	0.340	73.80%	0.630
	Weak staining	53.60%		78.60%	
	Strong staining	63.00%	0.420	67.40%	0.300
VEGF	Positive (47)	72.30%	0.003	83.00%	0.004
	Negative (27)	37.00%		51.90%	
	Low grade	41.90%		58.10%	
	High grade	72.10%	0.009	81.40%	0.030
	Weak staining	44.40%		60.00%	
	Strong staining	82.80%	0.001	89.70%	0.006

71.6% respectively. Two-year DFs and 2-year OS in patients with positive p-mTOR expression were 60% and 69.2% respectively which were not significantly different from patients with negative expression (P>0.05). Patients with positive VEGF expression showed significant improvement in two year OS and DFs: 72.3% and 83% (P<0.05) respectively (Table 2 and 3).

Subgroup analysis showed no significant difference between grading and intensity of p-mTOR expression and 2-year DFs and 2-year OS. High grading and strong VEGF expression were significantly higher than low grade and weak VEGF expression in 2-year DFs and 2-year OS (p<0.05) (Table 3).

Discussion

Ovarian clear cell carcinoma is a distinct histological subtype of epithelial ovarian cancer commonly diagnosed at an early stage. Nagaraja et al, reported that nearly 70% of clear cell ovarian carcinomas were diagnosed at early stage (I and II), which is similar to our study (74.4%) (Nagaraja AK et al. 2010).Clear cell carcinoma has a poor response to standard platinum-based chemotherapy regimen and a high recurrence with poor prognosis (Behbakht K et al. 1998; Sugiyama T et al. 2000; Pectasides D et al. 2006). Contrary to previous studies (Behbakht K et al. 1998; Sugiyama T et al. 2000; Pectasides D et al. 2006), our data showed that most of the cases (78.3%) were chemo-sensitive. This may be from a high proportion achieving of optimal surgical reduction (87.4%).

Reported malignant transformation of endometriosis ranged from 6.8% to 11% in previous study (Yagyu T et al. 2006). We found 29 cases (39.2%) where the carcinoma had arisen within or adjacent to endometriosis. We also found that p-mTOR and VEGF expression in the endometriosis associated group and no endometriosis group were similar (86.2% vs. 88.9% and 65.5% vs. 62.2% (p>0.05) respectively). This contrasted to a previously published study (Yagyu T et al. 2006). This may be that of menopausal status was not done in our study due to limited sample size.

To examine activity of p-mTOR and VEGF, either western blotting or immunohistochemistry (IHC) may be used. The IHC can show diffuse pattern and intensity of expression. Western blotting will works well for cells or tissue in which most of the cells have activated autophagy and cleavage. If all cells are not undergoing this process, the western blotting will not detect the signal and IHC is more useful. Paraffin-embeded specimens, showed few cells undergoing autophagy, and IHC was a suitable method for our study.

According to our study of 74 ovarian clear cell carcinoma patients, we found that 87.9% of the cases were positive for p-mTOR expression (83.6% in early stage and 100% in advance stage). This was similar to previous study. Mabuchi et al, studied in 52 ovarian clear cell carcinomas(CCC) and 46 ovarian serous adenocarcinomas(SEA) analyzed by clinical stage and found the frequency of strong phospho-mTOR immunoreactivity(86.4%) was significantly higher in clear cell carcinomas than in serous adenocarcinomas. Positive p-mTOR expression was significantly expressed in advanced stages (Mabuchi S et al. 2009).We also found that the FIGO staging was the independent prognostic factor for p-mTOR expression (p = 0.04).

The primary objective for this study is to determine the prevalence of p-mTOR and VEGF in ovarian clear cell carcinoma. We also analysed the correlations of the expressions with clinical characteristics and the prognostic factors, stage and chemo-sensitivity. Our study differed from the previous study (Altomare et al, 2004).The latter studied the phosphorylation of AKT on ovarian cancers in a tissue microarray and found over expression of p-AKT on 68% of the 31 tumors, Mabuchi et al, found that mTOR inhibition by RAD001 reduced human ovarian cancer cell proliferation, enhanced cisplatin-induced apoptosis and prolonged survival in an ovarian cancer xenograft model both in early stage and in advance stage (Mabuchi S et al. 2009). They evaluated the expression of p-mTOR by Western blotting and showed that RAD001 inhibited the expression of HIF 1 alpha and VEGF-A in vitro cell lines.

This study showed VEGF expression (63.5%), which was different from Miyazawa's study (88%) (Miyazawa M et al. 2009).This may be because of a highly dilution of anti-VEGF in our study (1:100) but may be no clinical significance. Miyazawa et al. examined mTOR, p-mTOR, HIF-1alpha and VEGF in surgically resected specimens of 29 SEA and 47 CCC. p-mTOR expression was more prominent in clear cell carcinomas than serous adenocarcinomas. No apparent relationship between p-mTOR expression and clinical features such as age or stage was noted. Successfully grown tumors had reduced considerably in size 2–3 weeks after treatment with everolimus (p-mTOR inhibitor). The effectiveness of anti-mTOR for treatment of CCC may be due to the successful inhibition of the mTOR-HIF-1alpha-VEGF sequence. But there has been no study regarding

correlations between mTOR and VEGF expression and clinical characteristics or survival. From our study, VEGF expression was significantly expressed in chemo-sensitive patient and found to be an independent prognostic factor ($p = 0.01$). Positive VEGF expression was also significant associate with increased 2-year disease free and overall survival. Subgroup analysis of high grade and strong VEGF expression demonstrated a higher percentage of 2-year DFs and OS. Therefore VEGF activity may be a prognostic factor which influenced in survival.

Trinh et al. showed that dual targeting of VEGF and mTOR in ovarian cancer xenograft models had antitumoural effect with survival benefit and was able to reverse the accumulation of ascites (Trinh et al. 2009). The targeted mechanism of anti-VEGF treatments in epithelial ovarian cancer is not only anti-angiogenic but also suppresses tumor cell growth factors acting as a AKT/mTOR signaling inhibitor on tumor cells. For this reason the correlation between p-mTOR and VEGF expression is provocative.

At our institution, this tumor was aggressive and had a higher recurrence rate compared to the serous counterpart. p-mTOR was frequently expressed in both advanced (100%) and early stage (83.6%) clear cell carcinomas with a trend to lower survival rates. mTOR seems to be a promising target for the treatment of patients with both early and advanced stage clear cell carcinoma and anti-mTOR compounds may become standard therapy in the future. In a phase II clinical trial (GOG170I),5 (9.3%) of 54 patients had partial response to temsirolimus (Torisel_) is a synthetic, ester analog of rapamycin monotherapy for refractory, recurrent ovarian, or primary peritoneal cancers(Behbakht K et al. 2011).A phase II study (GOG268) combining temsirolimus with carboplatin and paclitaxel following temsirolimus consolidation as first-line therapy is underway in patients with clear cell carcinoma of the ovary. Other mTOR inhibitors, such as everolimus (Afinitor_) and sirolimus, are also being evaluated in combination with bevacizumab or other cytotoxic agents (e.g. pegylated liposomal doxorubicin, carboplatin, docetaxel) in phase I and II settings (Itamochi H et al. 2012). Further study of incidence of p-mTOR in other types of ovarian carcinoma might be fruitful. Targeted therapy research regarding the combination of anti-mTOR and anti-VEGF treatment may be beneficial and subgroup analysis in chemo-sensitive and chemo-resistant patients should be performed.

In conclusion, ovarian clear cell carcinoma had a high prevalence of p-mTOR and VEGF expression. Although there was no correlation between p-mTOR and VEGF expression, they were significantly correlated with clinical characteristics and had prognostic significance in ovarian clear cell carcinoma patients. Further study of combinations of these targeted therapies in this area is warranted.

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