Original Article
Diagnostic value of beclin-1 and mTOR in ovarian cancer

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Abstract: This study described the expression of beclin-1 and mTOR proteins in 86 ovarian tumor tissue samples (n=25 benign ovarian tumor tissues; n=16 borderline ovarian tumor tissues; n=45 malignant epithelial ovarian tumor tissues) and 20 normal ovarian tissue samples and determined the diagnostic value of serum beclin-1 and mTOR protein levels for ovarian tumors. Serum and tissue beclin-1 and mTOR protein levels were detected with enzyme-linked immunosorbent assay, western blot, and immunohistochemistry. Receiver operating characteristic (ROC) curves were constructed to determine the significance of serum beclin-1 and mTOR protein levels for diagnosing borderline and malignant epithelial ovarian tumor. Mean beclin-1 protein levels were highest in normal ovarian tissue and were progressively decreased in benign, borderline, and malignant epithelial ovarian tumor. Mean mTOR protein levels were lowest in normal ovarian tissue and were progressively increased in benign, borderline, and malignant epithelial ovarian tumor. The area under the ROC curve was 0.93 or 0.97 when a combination of serum beclin-1 and mTOR protein levels was used to diagnose borderline or malignant epithelial ovarian tumor. There was a significant negative correlation between beclin-1 and mTOR protein expression in malignant epithelial ovarian tumor (r_s=-0.78, *P*<0.05), but no correlation between beclin-1 and mTOR protein expression in borderline ovarian tumor. Combined detection of serum beclin-1 and mTOR protein levels may have improved diagnostic accuracy for malignant epithelial ovarian tumor compared to each marker alone.

Keywords: Ovarian tumor, ovarian cancer, diagnostic value, beclin-1, mTOR

Introduction

Ovarian cancer is the most lethal gynecologic cancer. Globally, in 2018, there were 295,414 new cases of ovarian cancer and 184,799 deaths from the disease [1]. Ovarian cancer may go undetected until it is late stage and has spread within the pelvis and abdomen. Diagnosis of ovarian cancer at an early stage increases the potential for long-term survival after treatment.

Autophagy is a cellular self-digestion process that is essential for maintaining homeostasis in eukaryotes [2]. Autophagy has a complex role in cancer. During early stage tumorigenesis, autophagy contributes to tumor suppression. During late stage tumorigenesis, autophagy promotes malignant progression [3]. Detection of autophagy-related mechanisms may

have clinical utility for the diagnosis of ovarian tumors.

Beclin-1 is a protein that regulates autophagy and cell death through interactions with B-cell lymphoma-2 (BCL-2) or phosphatidylinositol-3-hydroxykinase (PI3k) class III. mTOR is a kinase that inhibits autophagy following activation by protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling. Studies describing the clinical significance of combined detection of serum beclin-1 and mTOR protein levels for diagnosis of malignant epithelial ovarian tumor are scarce. The objective of this study was to describe the expression of beclin-1 and mTOR protein in ovarian tumors and determine the diagnostic value of serum beclin-1 and mTOR protein levels for ovarian tumors. Beclin-1 and mTOR may represent new biomarkers for the early detection and monitor-

Table 1. Characteristics of the study population

	A	B	C	D	Total
Case number	20	25	16	45	106
Average age	43.5 ± 12.3	46.2 ± 13.1	42.8 ± 8.5	52.4 ± 10.4	-
Nationality (n)					
Han nationality	12	16	8	25	61
Bai nationality	5	6	4	13	28
Others	3	3	4	7	17
Preoperative time (d)	4.2 ± 1.8	3.9 ± 1.7	4.5 ± 2.3	3.7 ± 2.1	-

Group A, normal ovarian tissues; Group B, benign ovarian tumor tissues; Group C, borderline ovarian tumor tissues; Group D, malignant epithelial ovarian tumor tissues.

ing of disease progression in patients with ovarian cancer.

Materials and methods

In accordance with the journal's guidelines, we will provide our data for the reproducibility of this study in other centers if such is requested.

Subjects

This study included patients with ovarian tumors and patients with healthy ovarian tissue who underwent ovariectomy at the First Affiliated Hospital of Dali University between Nov. 2017 and Oct. 2019. Subjects mean age was 52 years (range, 19-73 years). Samples were divided into 4 groups: normal (Group A, normal ovarian tissues; n=20); benign (Group B, benign ovarian tumor tissues; n=25); borderline (Group C, borderline ovarian tumor tissues; n=16); and malignant (Group D, malignant epithelial ovarian tumor tissues; n=45). Metastasis was detected by computed tomography (CT) scan, ultrasonography, or observation during surgery (**Table 1**). The study was approved by the Ethical Board Committee of the First Affiliated Hospital of Dali University (20200714) and was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki. All included subjects provided written informed consent.

Sampling

Fasting venous blood samples (5 ml) were collected from all subjects in the morning 3 days before surgery. Sera were stored at -20°C until use. Ovarian tissue samples were collected during surgery. A portion of the tissue was stored at -80°C, and the remainder was formalin-fixed and paraffin-embedded following standard protocols.

Enzyme linked immunosorbent assay

Beclin-1 and mTOR protein were detected in serum using ELISA kits (Sun Red Biotechnology Company, Shanghai, China), according to the manufacturer's instructions. Serum beclin-1 and mTOR protein levels were calculated by measuring absorbance at a wavelength of 450 nm.

Western blotting

Total protein was isolated from tissue samples. 30 g protein was separated by electrophoresis on a 10% sodium dodecyl sulfate (SDS) gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in Tris-buffered saline containing 0.1% Tween-20 (TBST; 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) and 3% bovine serum albumin (BSA) for 3 h at room temperature to block nonspecific binding. Subsequently, the membrane was incubated with primary antibody against beclin-1 (1:500) or mTOR (1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. The membrane was washed three times with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, 1:5000; Cell Signaling Technology, Danvers, MA, USA). Protein bands were visualized using an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA). Band intensities were semi-quantified via densitometry analysis using Image-Pro Plus (IPP 6.0, Media Cybernetics, Maryland, U.S.). Relative protein expression was normalized to GAPDH.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were stained with hematoxylin and eosin to pre-

view the tissue and select sections suitable for immunohistochemistry. Sections were de-paraffinized in xylene, rehydrated in a series of graded alcohols, and autoclaved for five minutes in ethylene diamine tetra-acetic acid (EDTA). Endogenous peroxidase was quenched with 3% H_2O_2. Tissue sections were incubated with a primary antibody against beclin-1 (rabbit anti-human polyclonal antibody, 1:100; Abcam, UK) or mTOR (rabbit anti-human polyclonal antibody 1:100; Abcam, UK) overnight at 4°C. Sections were incubated with horseradish peroxidase conjugated streptavidin for 30 min and visualized with diaminobenzidine (DAKO, Glostrup, Denmark).

Immunohistochemistry was visually scored, as previously described [4], independently by two researchers who were blinded to the patients' information. Staining intensity was scored as 0, no staining; 1, faint staining; 2, moderate staining; or 3, strong staining. The distribution of labeled protein was defined as a percentage of the whole area and scored as: 0 (<5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (>75%). Total scores were determined by combining scores for staining intensity and distribution.

Beclin-1 and mTOR protein have a predominantly cytoplasmic staining pattern. The proportion of cells with strong beclin-1 and mTOR cytoplasmic expression was recorded in all available optical fields (×200; whole section), and a mean value was used to score each case.

Statistical analysis

Statistical analyses were performed with IBM SPSS version 20.0 software and GraphPad Prism version 7 software. Between group comparisons of median serum beclin-1 and mTOR protein levels and the rate of beclin-1 positive cells and mTOR positive cells in normal ovarian tissue, benign ovarian tumor tissue, borderline ovarian tumor tissue and malignant epithelial ovarian tumor tissue were performed with the Mann-Whitney U test. Correlation between serum beclin-1 and mTOR protein levels in 86 ovarian tumor tissue samples was assessed using Spearman's correlation test. The value of individual or combined detection of serum beclin-1 and mTOR protein levels in the diag-

nosis of malignant epithelial ovarian tumor was evaluated with receiver operating characteristic (ROC) curve and logistic regression analyses. Area under the curve (AUC), sensitivity, and specificity were calculated. Serum beclin 1 and mTOR protein levels were independent variables and the presence/absence of ovarian cancer was the dependent variable. A two-sided *p* value <0.05 was considered significant.

## Results

Serum beclin-1 and mTOR protein levels

Median serum beclin-1 protein levels in patients with normal ovarian tissue and benign ovarian tumor were not significantly different (*P*=0.945). Median serum beclin-1 protein levels were significantly lower in patients with borderline ovarian tumor compared to benign ovarian tumor (*P*=0.033), and in patients with malignant epithelial ovarian tumor compared to normal ovarian tissue (*P*<0.001), benign ovarian tumor (P<0.001), and borderline ovarian tumor (*P*<0.05).

Median serum mTOR protein levels in patients with normal ovarian tissue and benign ovarian tumor were not significantly different (*P*=0.918). Median serum mTOR protein levels were significantly higher in patients with borderline ovarian tumor compared to benign ovarian tumor (*P*<0.001), and in patients with malignant epithelial ovarian tumor compared to normal ovarian tissue (*P*<0.001), benign ovarian tumor (P< 0.001), and borderline ovarian tumor (*P*<0.05) (**Figure 1**).

Diagnostic value of serum beclin-1 and mTOR protein levels in ovarian tumor

Assessment of the value of individual or combination detection of serum beclin-1 and mTOR protein levels in the diagnosis of malignant epithelial ovarian tumor showed $AUC_{beclin-1 + mTOR}$ was 0.97, which was greater than AUC_{mTOR} (0.91) and $AUC_{beclin-1}$ (0.86). For borderline ovarian tumor, $AUC_{beclin-1 + mTOR}$ was 0.93, which was greater than AUC_{mTOR} (0.84) and $AUC_{beclin-1}$ (0.71). Individual or combined detection of serum beclin-1 and mTOR protein levels had no value for differentiating benign ovarian tumor from normal ovarian tissue (**Table 2**; **Figure 1**).
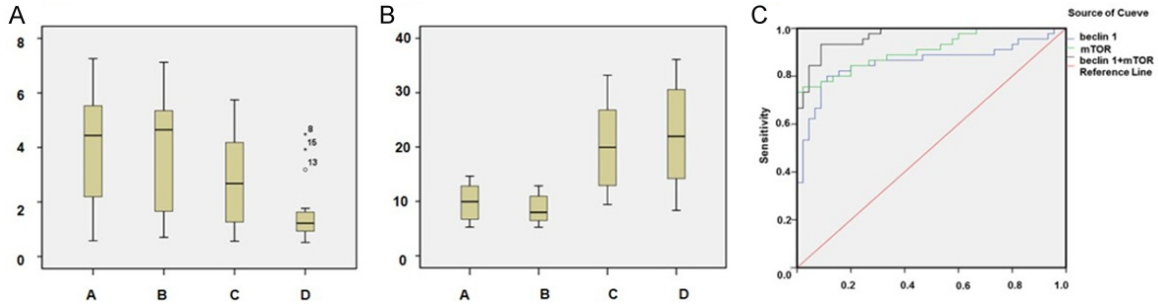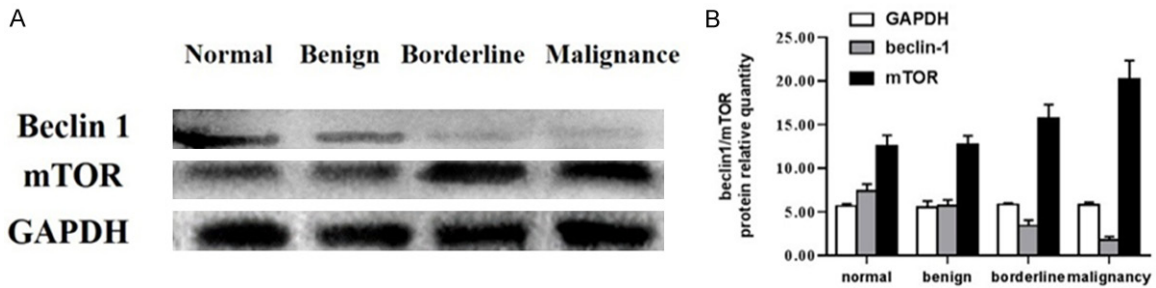
Figure 1. Serum beclin-1 and mTOR protein levels and ROC curve analysis. A. Serum beclin-1; B. Serum mTOR (Group A, normal ovarian tissues; Group B, benign ovarian tumor tissues; Group C, borderline ovarian tumor tissues; Group D, malignant epithelial ovarian tumor tissues); C. The diagnostic value of serum beclin-1 and/or mTOR protein levels (malignant epithelial ovarian tumor, $AUC_{beclin-1 + mTOR}$=0.97, AUC_{mTOR}=0.91, $AUC_{beclin-1}$=0.86).

Table 2. Diagnostic value of serum beclin-1 and mTOR protein levels in ovarian tumors

	Epithelial ovarian tumor			Borderline ovarian tumor		
	Beclin-1	mTOR	Beclin-1 + mTOR	Beclin-1	mTOR	Beclin-1 + mTOR
AUC	0.86	0.91	0.97	0.71	0.84	0.93
95% CI	0.77-0.94	0.85-0.97	0.94-1.00	0.55-0.88	0.70-0.97	0.83-1.00
P-value	<0.001	<0.001	<0.001	0.029	0.001	<0.001
Youden index	0.689	0.734	0.689	0.4	0.58	0.79
Sensitivity (%)	80	75.6	77.8	75	62.5	93.75
Specificity (%)	88.9	97.8	91.1	65	95	85
Critical values	1.809	18.27	-	3.96	17.13	-
Positive predictive value (%)	81.6	97.1	97.3	63.16	90.91	83.33
Negative predictive value (%)	87.8	80	83	76.47	76	94.44

Figure 2. Beclin-1 and mTOR protein levels in ovarian tumor tissue. Tissue lysates were analyzed for beclin-1, mTOR, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein levels. Data represent normalized protein expression from triplicate experiments.

Beclin-1 and mTOR protein levels in ovarian tumor tissue

Mean beclin-1 protein levels were highest in normal ovarian tissue and were progressively decreased in benign ovarian tumor, borderline ovarian tumor, and malignant epithelial ovarian tumor.

Mean mTOR protein levels were lowest in normal ovarian tissue and were progressively increased in benign ovarian tumor, borderline ovarian tumor, and malignant epithelial ovarian tumor (**Figure 2**).

Immunohistochemistry

Beclin-1 and mTOR protein appeared as brown, yellow or tan cytoplasmic staining. The rate of beclin-1 positive cells was significantly lower in borderline ovarian tumor and malignant epithelial ovarian tumor compared to nor-

Table 3. Beclin-1 and mTOR protein expression in ovarian tissue

Group (n)	Beclin-1			mTOR		
	Positive	Negative	Positive rate (%)	Positive	Negative	Positive rate (%)
Group A (20)	17	3	85.0	6	14	30.0
Group B (25)	19	6	76.0	9	16	36.0
Group C (16)	5	11	31.3	12	4	75.0
Group D (45)	13	32	28.9	37	8	82.2
P	<0.001 (24.23)			<0.001 (24.33)		
$P^{A\ vs\ B}$	0.453 (0.56)			0.672 (0.18)		
$P^{A\ vs\ C}$	0.001 (10.81)			0.007 (7.20)		
$P^{A\ vs\ D}$	<0.001 (17.54)			<0.001 (16.86)		
$P^{B\ vs\ C}$	0.005 (8.05)			0.015 (5.94)		
$P^{B\ vs\ D}$	<0.001 (14.37)			<0.001 (15.24)		
$P^{C\ vs\ D}$	0.859 (0.03)			0.533 (0.39)		
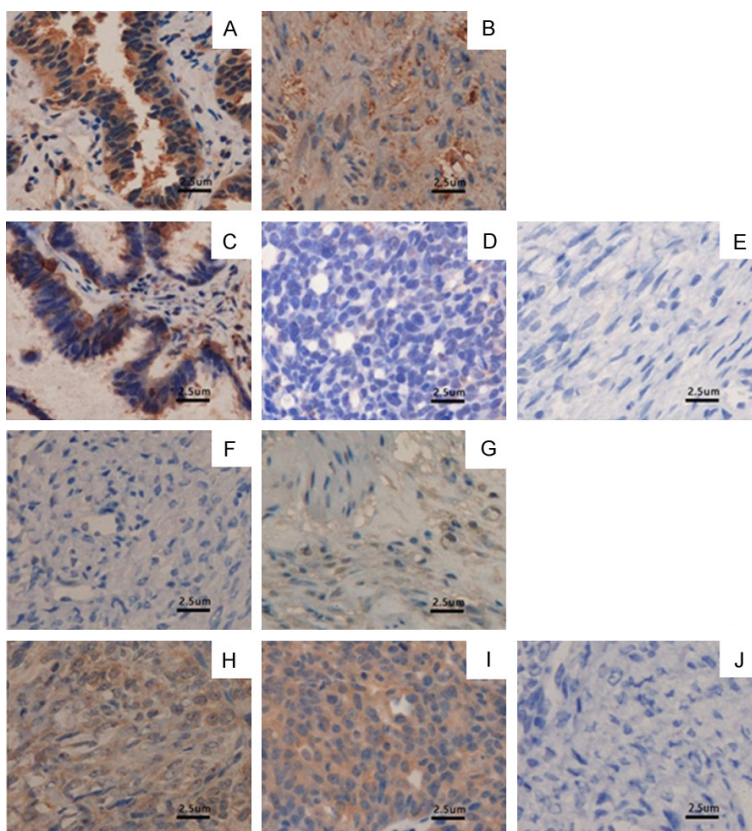
Figure 3. Proteins beclin-1 and mTOR expression in ovarian tumor. A. Beclin-1 in normal ovarian tissue; B. Beclin-1 in benign ovarian tumor tissue; C. Beclin-1 in borderline ovarian tumor tissue; D. Beclin-1 in malignant epithelium; E. Negative control; F. mTOR in normal ovarian tissue; G. mTOR in benign ovarian tumor tissue; H. mTOR in borderline ovarian tumor tissue; I. mTOR in malignant epithelial ovarian tumor tissue; J. Negative control (400×).

The rate of mTOR positive cells was significantly higher in borderline ovarian tumor and malignant epithelial ovarian tumor compared to normal ovarian tissue or benign ovarian tumor ($P \leq 0.007$) (**Table 3**; **Figure 3**).

Clinical relevance of beclin1 and mTOR protein expression in malignant epithelial ovarian tumor

Spearman correlation analysis revealed a significant negative correlation between beclin-1 and mTOR protein expression (percentage of positive cells) in malignant epithelial ovarian tumor (r_s=-0.78, P<0.05). There was no correlation between beclin-1 and mTOR protein expression in borderline ovarian tumor (r_s=-0.45, P>0.05).

Discussion

An unmet clinical need exists for an effective screening strategy for ovarian cancer that has a high sensitivity for early-stage disease, which is usually asymptomatic. An estimated 60% of women with ovarian cancer are diagnosed at an advanced stage, which generally translates to poor prognosis [5].

mal ovarian tissue or benign ovarian tumor ($P \leq 0.005$).

Basal autophagy has a housekeeping role in most cell types where it maintains the integrity of intracellular organelles and proteins. Autophagy is upregulated as an adaptive response to intra- or extracellular stress, including starvation, infection, or oxidative stress, and as a result of oncogene activation during the progression of cancer [6]. Dynamic alterations in autophagy activation can be detected by monitoring changes in autophagy-related markers, such as beclin-1 and mTOR. Targeting these autophagy-related markers may yield significant clinical benefits for patients with ovarian cancer.

Beclin-1, located on chromosome 17q21, is a mammalian ortholog of the yeast autophagy-related gene 6 (Atg6) [7]. Targeted mutant mouse models demonstrated that beclin-1 is a haplo-insufficient tumor-suppressor gene. Specifically, beclin-1-/- mutant mice died early in embryogenesis and beclin-1+/- mutant mice suffered from a high incidence of spontaneous tumors [8], and heterozygous disruption of beclin-1 increased the frequency of spontaneous malignancies and accelerated the development of hepatitis B virus-induced premalignant lesions [9].

Abnormal beclin-1 expression in tumor tissues is associated with the pathogenesis of many cancers. High beclin-1 expression may represent a favorable prognostic factor in colorectal cancer [10] and oral squamous cell carcinoma [11], but an adverse prognostic factor in endometrioid adenocarcinoma [12], nasopharyngeal carcinoma [13], and pancreatic ductal adenocarcinoma [14]. The beclin-1 gene is mono-allelically deleted in up to 75% of ovarian cancers, where low beclin-1 expression predicts an unfavorable response to platinum-based chemotherapy [9].

Serum beclin-1 protein levels are altered during the development of various disease conditions. In patients with diabetic kidney disease (DKD), serum beclin-1 protein levels were reduced, related to the stage of DKD, and correlated with the degree of albuminuria [15]. In patients with ulcerative colitis, serum beclin-1 protein levels were increased [16]. To the authors' knowledge, the present study is the first to describe serum beclin-1 protein levels in patients with ovarian tumors. Median serum beclin-1 protein levels were lowest in patients wi-

th malignant epithelial ovarian tumor and progressively increased in patients with borderline ovarian tumor, benign ovarian tumor, and normal ovarian tissue.

Numerous studies have reported on beclin-1 protein expression in ovarian tumors. Using immunohistochemistry, Lin et al. showed the rate of beclin-1 positive cells was 97.1% in normal ovarian tissue, 84.6% in benign cystadenoma, 80% in borderline ovarian tumor, and 44.4% in epithelial ovarian tumor [17]. In the present study, immunohistochemistry revealed the rate of beclin-1 positive cells was 85.0% in normal ovarian tissue, 76.0% in benign ovarian tumor, 31.3% in borderline ovarian tumor, and 28.9% in epithelial ovarian tumor.

mTOR is a serine/threonine protein kinase that is a core component of the PI3K/AKT/mTOR signaling pathway that represses autophagy [18]. Accumulating evidence suggests that mTOR dysfunction contributes to cancer [19]. Genetic alterations in the PI3K/AKT/mTOR pathway have been detected in 36% of small cell lung tumors from Japanese patients [20], 42% of primary prostatic tumors and all metastatic tumors [21], and high-grade serous ovarian cancer [22]. High expression of mTOR in ovarian cancer may promote cell cycle progression and tumor cell proliferation [23]. In a previous study, 87% of clear cell ovarian carcinomas had elevated mTOR activity, while inhibition of the mTOR pathway sensitized these cells to cisplatin [24]. In another report, the expression of mTOR was obviously increased in advanced ovarian cancer [25]. In the present study, median serum mTOR protein levels were highest in patients with malignant epithelial ovarian tumor and progressively decreased in patients with borderline ovarian tumor, benign ovarian tumor, and normal ovarian tissue.

Several studies used immunohistochemistry to investigate the expression status of mTOR in ovarian tissues [26]. Yang et al. showed minimal mTOR expression in benign ovarian tumor and normal ovarian tissue. Xie et al. reported the rate of mTOR positive cells was 23.2% in normal ovarian tissue, 50.0% in benign ovarian tumor, and 68.5% in epithelial ovarian tumor [27]. Shang [28], and Ghoneum [29] showed that the PI3K/AKT/mTOR pathway was associated with aggressive disease, resis-

tance to therapy, recurrence, and a poor prognosis in patients with ovarian cancer. In the present study, immunohistochemistry revealed the rate of mTOR positive cells was 30.0% in normal ovarian tissue, 36.0% in benign ovarian tumor, 75.0% in borderline ovarian tumor, and 82.2% in epithelial ovarian tumor.

These data imply that disruptions of autophagy may be a source of pathogenesis in ovarian cancer, and that beclin-1 and mTOR activity may be related to the pathological characteristics of ovarian tumors. Consistent with this, combined beclin-1 and mTOR expression may represent a biomarker for the evaluation of ovarian tumors. Previous studies show beclin-1 and mTOR protein expression are negatively correlated in breast and non-small cell lung cancer [30, 31], and findings from the present study showed a negative correlation between serum beclin-1 and mTOR protein levels in patients with malignant epithelial ovarian tumor.

The sensitivity and specific of autophagy markers in the diagnosis of various diseases differs. Previous reports show mTOR could diagnose IgA nephropathy at a cut-off value of 0.930 (relative gene expression level) with a sensitivity of 90.2% and a specificity of 73.8% and renal fibrosis at a cut-off value of 0.301 with a sensitivity of 71.7% and a specificity of 64.8% [32], mTORC1 could diagnose preeclampsia at a cut-off value of 2113.35 ng/mL with a sensitivity of 93.5% and a specificity of 70.1% [33], and mTOR in cervical lesions could predict malignancy with a sensitivity of 94.0% and a specificity of 60.0% [34]. In the present study, serum beclin-1 protein levels alone could diagnose malignant epithelial ovarian tumor with a sensitivity of 81.6% and a specificity of 88.9%, serum mTOR protein levels alone could diagnose malignant epithelial ovarian tumor with a sensitivity of 75.6% and a specificity of 97.8%, and a combination of serum beclin-1 and mTOR protein levels could diagnose malignant epithelial ovarian tumor with a sensitivity of 77.8% and a specificity of 91.1%, a positive predictive value of 97.3% and a negative predictive value of 83.0%. These data suggest that combined detection of serum beclin-1 and mTOR protein levels may have improved diagnostic accuracy for malignant epithelial ovarian tumor compared to each marker alone.

In conclusion, this preliminary analysis described the expression of beclin-1 and mTOR protein in serum and tissue from patients with ovarian tumors. Findings suggest that autophagy may be a source of pathogenesis in ovarian cancer. Further studies are required to elucidate the molecular mechanisms underlying these data, and their clinical significance.

Disclosure of conflict of interest

None.

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