Alteration and clinical relevance of PTEN expression and its correlation with survivin expression in epithelial ovarian tumors

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Abstract. The tumor suppressor PTEN, phosphatase and tensin homolog on chromosome 10, plays an essential role in regulating signaling pathways involved in cell growth and apoptosis and is inactivated in a wide variety of tumors. Survivin, a member of the inhibitor of apoptosis protein family (IAP), is associated with cell proliferation, and overexpressed in common human tumors. Both PTEN and survivin proteins can regulate cell cycle and apoptosis, but their biological effects are adverse. We have previously investigated the role of survivin expression in epithelial ovarian tumors. In this study, we evaluated the alteration and clinical relevance of PTEN expression and further assessed its correlation with survivin expression in epithelial ovarian tumors. Immuno-histochemical analysis was performed in 103 cases of ovarian tumors, and 26 of the 103 cases were evaluated by Western blot analysis. PTEN expression was reduced from benign to malignant ovarian tumors ($p=0.0003$), and an inverse correlation between PTEN and survivin was found in benign, borderline, and malignant tumors ($p=0.004$, $p=0.015$ and $p=0.0005$, respectively). PTEN expression was significantly associated with tumor grade ($p=0.001$), histological subtype ($p=0.037$), ascites ($p=0.038$), and residual disease ($p=0.0006$). Kaplan-Meier survival analysis showed that the loss of PTEN expression was significantly associated with poor overall survival ($p=0.021$), and patients with PTEN(-)/survivin(+) expression had the worst prognosis among all phenotypes of PTEN/survivin expression ($p=0.039$). Our results suggest that the altered PTEN expression and its inverse correlation with survivin may be involved in the development and progression of ovarian tumors, and the combined detection of PTEN and survivin proteins might be more valuable in the evaluation of malignancy and prognosis in epithelial ovarian tumors.

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Introduction

PTEN, phosphatase and tensin homolog on chromosome 10, was highlighted as a tumor suppressor gene (1,2), which encodes a dual activity phosphatase and is centrally placed to regulate a network of multiple signal transduction pathways involved in cell cycle regulation and cell adhesion properties, including cell proliferation, migration and death (3). PTEN can inhibit cell cycle progression and induce G_1 arrest through negatively regulating the PI3K (phosphatidylinositol 3-kinase)/AKT (protein kinase B) signaling pathway (4-7). A loss of PTEN function leads to increased PI3K/AKT activity and subsequent increased cell proliferation, reduced apoptosis, altered migration, and increased size in all phenotypes that favor tumorigenesis (8). PTEN mutations or deletions have been identified in a large number of primary malignant tumors including glioblastomas, and breast, gastric, esophageal and endometrial cancer, and also showed that reduced PTEN protein was associated with poor prognosis (9-15). The PI3K/AKT pathway is a major driving force in human cancer, and a common way of stimulating the PI3K/AKT pathway occurs through inactivation of the PTEN tumor suppressor. PI3K has been implicated not only in cell survival signaling, but also in the inhibition of apoptosis by inactivation of cell death proteins such as BAD14, and a death effector protein, caspase-9 (16).

Survivin, a member of the IAP family, is found to be expressed in fetal tissues and many cancers but undetectable in most normal adult tissues (17). Survivin can suppress apoptosis induced by Fas, Bax, caspases, and anticancer drugs (18). Our study (19) and other studies (20-25) have indicated that survivin is associated with the aggressiveness of tumors and unfavorable clinical outcomes. Additionally, Kim *et al* have reported that inhibition of the PI3K/AKT pathway down-regulates survivin expression in neuroblastomas (26), and activation of the PI3K/AKT pathway can increase survivin expression (27-30). These findings raised the question whether increased survivin expression in ovarian carcinoma is related to reduced PTEN expression. To gain better insight, we investigated the correlation of PTEN and survivin expression, as well as the clinical outcomes of PTEN in a group of epithelial ovarian tumors.

Materials and methods

Tumor specimens. Formalin-fixed, paraffin-embedded blocks of ovarian tumor tissues from 77 patients (32 malignant, 23
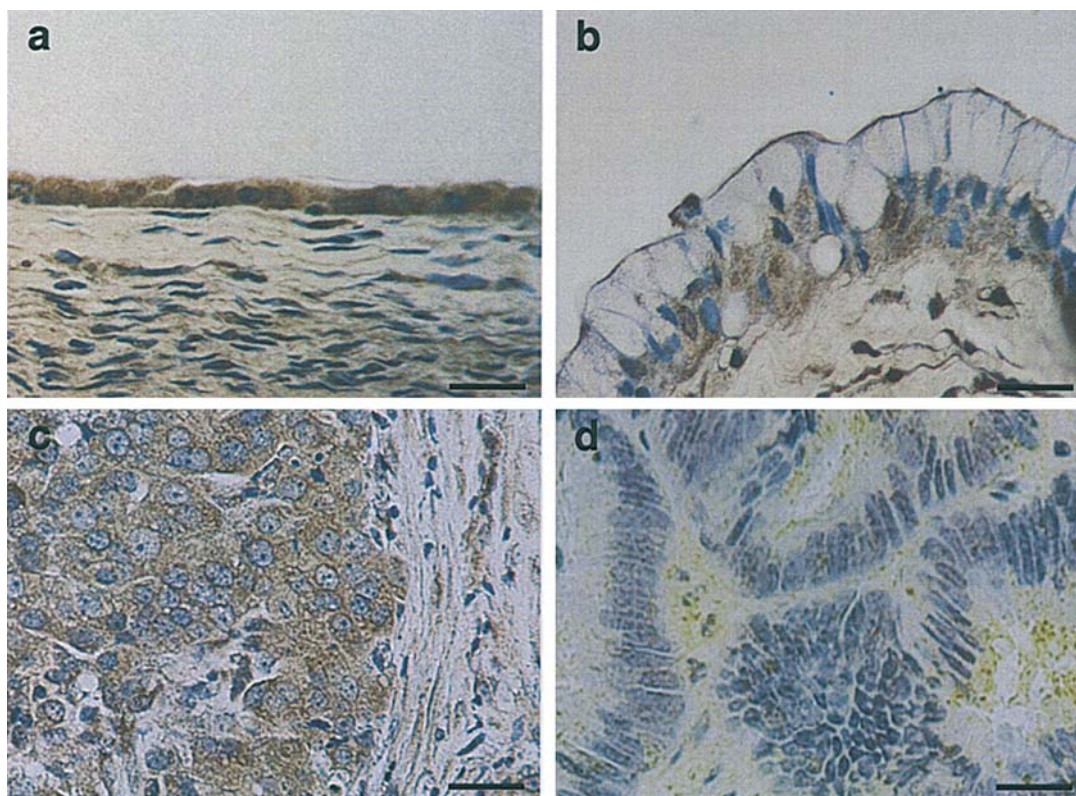
Figure 1. Immunohistochemical staining patterns of PTEN in ovarian tumors. (a) Positive staining was shown in benign ovarian tumor cells. (b) Positive staining was shown in borderline ovarian tumor cells. (c) Positive staining was shown in ovarian carcinoma cells. (d) Negative staining was shown in ovarian carcinoma cells. Scale bars, 30 μm.

borderline, and 22 benign ovarian tumors) were obtained at the Department of Perinatology and Gynecology, Faculty of Medicine, Kagawa University from 1985 to 1996. Fresh ovarian tumor samples (n=26; 15 malignant and 11 benign ovarian tumors) were obtained at the Department of Perinatology and Gynecology, Faculty of Medicine, Kagawa University and Department of Obstetrics and Gynecology of Takamatsu Red Cross Hospital from 1997 to 1998. After surgical resection, each fresh tumor specimen was immediately washed and cut out around necrotic tissue, then divided into two portions: one was instantly frozen for protein extraction and the other was formalin-fixed and paraffin-embedded for routine and immunohistochemical investigation. Specimens consisted of 33 benign, 23 borderline and 47 malignant ovarian tumors. The median age of the 47 ovarian carcinoma patients was 49 years (range, 16-77), and 16 patients were in stage I, 3 in stage II, 16 in stage III, and 12 in stage IV according to the International Federation of Gynecology and Obstetrics (FIGO) classification. The histological classification of tumors was carried out according to the WHO system with 21 well-differentiated (G_1), 13 moderately differentiated (G_2) and 13 poorly differentiated (G_3, including 2 undifferentiated) cases. Among the 47 patients with ovarian carcinomas, none received preoperative chemotherapy or radiotherapy. All patients received post-operative, platinum-based chemotherapy, but no radio-therapy. Follow-up data were available for all patients.

Immunohistochemistry. Paraffin sections (4 μm thickness) were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide (30 min).

To reduce nonspecific binding, the sections were incubated with 10% horse serum for 60 min at room temperature. The antigen retrieval procedure was performed by microwave oven heating, 3 times for 3 min each in 10 mM citric acid at pH 6.0. The sections were incubated overnight at 4˚C with mouse anti-PTEN monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100), and a corresponding section for each case was incubated with nonimmunized mouse serum as a negative control. Immunostaining was performed by the ABC (avidin-biotin peroxidase complex) method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was detected using DAB (3,3-diaminobenzidine) as the chromogen and Mayer's hematoxylin as the counterstain. All of the samples (formalin-fixed and fresh) were handled using the same method and antibody.

The immunoreactive cells were independently evaluated by two of our members (L.S. and Y.D.), who were unaware of the clinicopathological factors and clinical outcome of patients. At least 10 high-power fields were chosen randomly, and 1000 cells were counted. PTEN immunoreactivity was estimated according to the cytoplasmic staining intensity and percentage of positivity. The staining intensity was divided into 3 categories: 0, absent; 1, weak; and 2, moderate to strong. The quantity of stained cells was scored as: 1, <10%; 2, 10-50%; and 3, >50%. A total score of ≤4 was defined as reduced PTEN expression and denoted as (-).

Western blot analysis. Approximately 0.5 g of tissue from each fresh tumor sample was homogenized and lysed in 2.5 ml of lysis buffer [1% NP-40, 150 mM NaCl, 50 mM NaF, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM Na_3VO_4,

Table I. PTEN expression in benign, borderline, and malignant ovarian tumors.

| | | PTEN expression | | |
	Total	+ (%)	- (%)	p-value
Benign	33	29 (87.9)	4 (12.1)	<0.0001[a]
Borderline	23	16 (69.6)	7 (30.4)	0.0500[b]
Malignant	47	21 (44.7)	26 (55.3)	0.0003

[a]Benign vs. malignant. [b]Borderline vs. malignant.
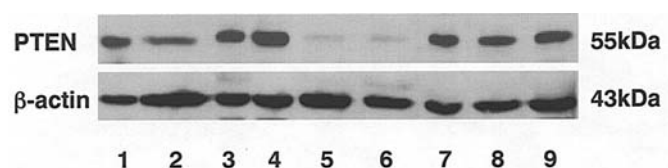
Figure 2. Western blot analysis of PTEN in benign and malignant ovarian tumors. PTEN expression was observed in all benign tumors (lanes 1-4). General or reduced PTEN expression was observed in malignant tumors (lanes 5-8). NGF-treated PC12 cells were used as a positive control (lane 9) (43). The amount of ß-actin was demonstrated at a constant level among the samples.

$10\ \mu M\ Na_2MnO_4$, 1 mM PMSF, 10 μg/ml leupeptin, and 1% aprotinin]. The lysates were centrifuged at 100,000 x g for 1 h at 4°C and the supernatant was stored at -80°C until further analysis. Extracts equivalent to 200 μg of the total protein were separated by 12% SDS-polyacrylamide gel, then transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked in TBS containing 5% nonfat dried milk, 10% sheep serum and 0.1% Tween-20, then probed by monoclonal antibody against PTEN (1:200), and against ß-actin (1:500; Sigma, St. Louis, MO, USA) in PBS containing 5% bovine serum. After several washes with TBS, membranes were probed with a horseradish peroxidase-conjugated anti-mouse IgG (Dako, Kyoto, Japan), and proteins were detected using an enhanced chemiluminescence (ECL) system (Amersham, Tokyo, Japan).

Statistical analysis. The correlation between PTEN and clinicopathological parameters was assessed using Pearson's χ^2 test. The Spearman rank correlation was used to determine whether there was a correlation between PTEN and survivin expression. Overall survival was calculated using the Kaplan-Meier method, and comparison between groups was performed with the log-rank test. The Cox proportional hazards regression model was used to estimate the relative risk ratio (RR) of death in the 95% confidence interval (CI) and identify the variables associated with overall survival. Statistical significance was set at $p<0.05$. Statistical analyses were run using JMP software version 3.2.5 (SAS Institute Inc., Cary, NC).

Results

Expression of PTEN in ovarian tumors. Immunohistochemical analysis revealed that PTEN expression was located mainly

Table II Correlation between PTEN expression and clinico-pathological parameters.

| | | PTEN expression | |
Parameters	Total	(%)	p-value
Age			
≤60	37	17 (46.0)	
>60	10	4 (40.0)	0.7370
Stage			
I-II	19	11 (57.9)	
III-IV	28	10 (35.7)	0.1330
Grade			
G_1	21	15 (71.4)	
G_2	13	5 (38.5)	
G_3	13	1 (7.7)	0.0010
Histology			
Serous	20	6 (30.0)	
Mucinous	14	11 (78.6)	
Endometrioid	6	1 (16.7)	
Clear cell	5	3 (60.0)	0.0370[a]
Undifferentiated	2	0 (0.0)	0.0370
Lymph node			
Negative	19	10 (52.6)	
Positive	24	8 (33.3)	0.2030
Ascites			
Negative	15	10 (66.7)	
Positive	32	11 (34.4)	0.0380
Residual disease			
≤2 cm	25	17 (68.0)	
>2 cm	22	4 (18.2)	0.0006

[a]Does not include undifferentiated type.

in the cytoplasm of ovarian tumor cells (Fig. 1a-c), and weak nuclear staining was also shown; an example of negative expression is shown in Fig. 1d. Reduced PTEN expression was detected in 12.1% (4 of 33) of benign tumors, 30.4% (7 of 23) of borderline tumors, and 55.3% (26 of 47) of malignant tumors (Table I). The PTEN expression in malignant tumors was significantly lower than that in benign tumors (p<0.0001), and borderline lower than that in borderline tumors (p=0.05). The total tendency of PTEN expression was decreased from benign to borderline to malignant tumors (p=0.0003).

To confirm the specificity of the immunohistochemical results, Western blot analysis was carried out in 11 benign and 15 malignant ovarian tumors, in which freshly frozen materials were available. An example of Western blot analysis is shown in Fig. 2. An immunoreactive band of PTEN was observed in both benign and malignant ovarian tumors (lanes 1-4, 7 and 8), and 2 cases of malignant tumor showed reduced PTEN expression (lanes 5 and 6).

Correlation of PTEN expression with survivin. Our previous data showed survivin expression in 21.2% of benign, 47.8%
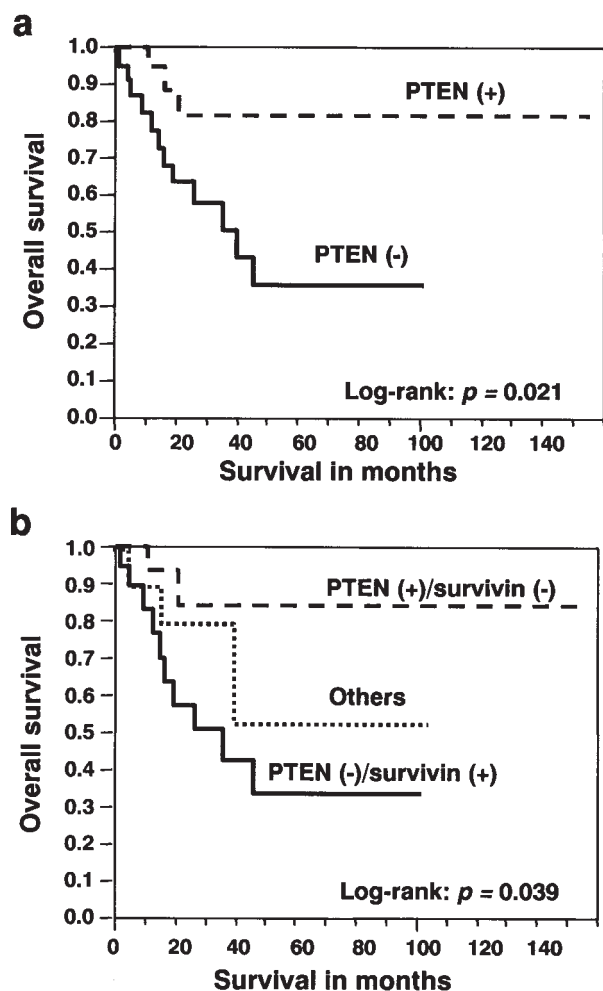
a

b

Figure 3. Kaplan-Meier survival curves of patients with ovarian carcinoma. (a) Overall survival according to PTEN expression (p=0.021). (b) Overall survival according to PTEN/survivin phenotypes (p=0.039).

in borderline, and 51.1% in malignant tumors, respectively (19). The correlation between PTEN and survivin expression was investigated using Spearman's rank correlation. A negative correlation between both proteins was identified in benign (correlation coefficient, -0.49; p=0.004), borderline (correlation coefficient, -0.5; p=0.015), and malignant tumors (correlation coefficient, -0.49; p=0.0005). Patients with PTEN(-)/survivin(+) expression showed 9.1%, 26.1% and 42.6% benign, borderline, and malignant tumors, respectively, and the ratio was increased with malignancy (p=0.005). In contrast, the patients with PTEN(+)/survivin(-) expression showed 75.8%, 47.8%, and 36.2% benign, borderline, and malignant tumors, respectively, and the ratio showed a decreasing tendency (p=0.002).

Correlation between PTEN expression and clinicopathological parameters. We evaluated the correlation between PTEN expression and clinicopathological parameters such as tumor grade, clinical stage, histology, lymph node status, etc. A significant correlation between PTEN expression and tumor grade (p=0.001), histology (p=0.037), ascites (p=0.038), and residual disease (p=0.0006) was revealed (Table II).

Survival analysis. The median follow-up time for all of the ovarian carcinoma patients was 24 months (range, 2-156). At the end-point of the follow-up, 32 patients survived with a median follow-up time of 27.5 months (range, 4-156), and 15 had died of ovarian cancer after a median follow-up of 17 months (range, 2-46). Kaplan-Meier analysis showed that reduced PTEN expression has a significant adverse effect on overall survival (p=0.021; Fig. 3a). Our previous study showed that increased survivin expression was significantly associated with poor overall survival (p=0.037) (19). In the current study, we further analyzed the combined phenotypes of PTEN and survivin proteins. The patients with PTEN(-)/survivin(+) expression had the worst overall survival among all phenotypes of PTEN/survivin (p=0.039; Fig. 3b).

Multivariate Cox regression analysis of PTEN expression was performed together with other factors showing prognostic significance of univariate analysis such as clinical stage (RR, 1.98; CI, 1.11-4.16; p=0.019), tumor grade (RR, 1.94; CI, 1.13-3.70; p=0.015), lymph node status (RR, 2.41; CI, 1.25-6.15; p=0.007), and residual disease (RR, 2.79; CI, 1.57-5.87; p=0.0003). Although PTEN expression was not defined as an independent prognostic factor, the patients with reduced PTEN expression have the highest relative risk ratio of death (RR, 3.96; CI, 1.26-7.41; p=0.017).

Discussion

In the present study, PTEN expression was found in 87.9%, 69.6% and 44.7% of benign, borderline, and malignant ovarian tumors, respectively. A significant difference was observed between benign and malignant ovarian tumors (p<0.0001). PTEN expression was gradually diminished from benign to borderline to malignant tumors (p=0.0003). The finding suggested that down-regulated PTEN expression might be closely associated with malignant transformation of ovarian tumors. Although no immunohistochemical data were available for PTEN expression simultaneously in benign, borderline, and malignant ovarian tumors, a correlation between carcinogenesis and a lack of PTEN expression has been demonstrated in ovarian carcinoma (31,32) and other cancers. Diminished or absent PTEN expression was reported in 66% of endometrial carcinoma (33), 38% of breast cancer (34), 27.2% of prostate cancer (35), and 24% of non-small cell lung cancer (36). Consequently, the alteration of PTEN expression might be a major general event in the progression of carcinomas of different origins.

Survivin, an inhibitor of apoptosis protein, was investigated in our previous study, and found to play a crucial role in the progression of ovarian carcinomas (19). It is recognized that PI3K/AKT pathway-dependent cell survival is negatively regulated by PTEN (8,37). PTEN and survivin are two inverse factors of apoptosis; thus, we attempted to investigate whether PTEN and survivin have a relationship in ovarian tumors. It was firstly found that PTEN expression was negatively correlated with survivin expression in the progression of ovarian tumors, and cases with PTEN(-)/survivin(+) expression gradually increased from benign to malignant tumors (p=0.005); in contrast, cases with PTEN(+)/survivin(-) expression gradually decreased from benign to malignant tumors (p=0.002). Previous studies

(26-30) revealed that the PI3K/AKT pathway could modulate apoptosis by a mechanism involving the PI3K/AKT pathway activation, subsequent up-regulation of survivin, and the suppression of caspase-9 activity called the AKT/survivin pathway (28,30). PTEN is known to regulate the PI3K/AKT pathway by removing the third phosphate from the inositol ring of the PIP3 second messenger (38-41), consequently inactivating the PI3K/AKT signaling pathway. From this, we can presume that PTEN might modulate the survivin level by the PI3K/AKT pathway. Ovarian tumors lacking PTEN expression have high survivin expression, thus intensifying the cell survival signal transduction pathway and promoting the progression of ovarian tumors.

In addition, we evaluated the correlation between PTEN expression and clinicopathological parameters. Reduced PTEN expression was found to be significantly associated with increasing tumor grade, ascites, and residual disease. The results are supported by the fact that mammalian cells lacking PTEN can promote proliferation, reduce apoptosis, alter migration, and increase size, which might be responsible for conferring the local invasion of tumors (8). We also found that PTEN expression was significantly lower in the endometrioid histological subtype than other subtypes, which is consistent with the report of Obata *et al*, who documented that lack of PTEN expression was frequently observed in endometrioid but not serous or mucinous ovarian carcinomas (42). Our finding therefore supports that the molecular pathogenic mechanism of endometrioid ovarian carcinoma is different than other histological subtypes, and PTEN might play an important role in the etiology of the endometrioid subtype.

The possible role of PTEN protein on the prognosis of ovarian tumor patients remains unclear. Currently, prognostic evaluation is primarily based on the traditional method that includes clinical stage, tumor grade, lymph node status, etc. However, in past decades, tumor markers have been demonstrated to be clinically helpful in detecting various human tumors. Studies on PTEN expression in breast, brain, gastric, esophageal, and endometrial cancer indicated that reduced PTEN protein was associated with poor prognosis (9-15). In this study, survival analysis by the Kaplan-Meier method revealed that patients lacking PTEN expression have a significantly shorter overall survival and show an approximate 4-fold higher relative risk of death (RR, 3.96; p=0.017). This evidence suggests that a low PTEN level confers a more aggressive phenotype of ovarian tumor cells and indicates that evaluation of the PTEN level may provide a significant prognostic implication in epithelial ovarian carcinomas. Schondorf *et al* have demonstrated that declining PTEN expression results in a shortened progression-free interval by assessing the PTEN content in both the primary and recurrent ovarian cancer specimens from each patient (32). We also performed a survival analysis of survivin in epithelial ovarian tumors, and found that patients with survivin overexpression had a significantly decreased overall survival (19). To verify whether the combined analysis of PTEN and survivin can provide a more valuable implication in the prognostic evaluation of ovarian carcinoma, a combined phenotype of these two proteins was further examined. We found that patients with PTEN(-)/survivin(+) expression had the worst overall survival among all phenotypes of PTEN/

survivin expression. Since the clinical outcome of ovarian carcinoma patients might be difficult to predict, the combined evaluation of PTEN/survivin can further increase the availability of prognostic information, and the identification of tumors with specific immunophenotypes may prove valuable in the future when selecting patients for experimental treatment protocols.

In conclusion, PTEN expression is negatively associated with survivin expression in the progression of ovarian tumors, and the alteration of PTEN expression is likely an important molecular event in ovarian tumorigenesis. Although PTEN expression cannot be recognized as an independent prognostic factor, the immunohistochemical evaluation of PTEN expression may provide additional prognostic information. Furthermore, the combined detection of PTEN/survivin proteins might be the most reliable indication of prognosis for epithelial ovarian carcinoma patients.

Acknowledgements

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