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Original Article

Overexpression of β-catenin and cyclinD1 predicts a poor prognosis in ovarian serous carcinomas

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Abstract: Ovarian serous cancer is the most common subtype of epithelial ovarian cancer, and is the leading cause of death from gynecologic cancer. There is an important need for exploration of diagnostic and prognostic markers for this disease. β-catenin and cyclinD1 play central roles in the tumorigenesis for certain cancers. The role of β-catenin and cyclinD1 in diagnosis and prognosis of ovarian serous carcinoma is uncertain. In the present study, the expression of β-catenin and cyclinD1 was examined in 60 ovarian serous carcinomas patients with immunohistochemical staining. The relationship between expression of β-catenin and cyclinD1 and FIGO stage, pathological grade was analyzed. Kaplan-Meier survival function was used to analyze the prognosis. Overexpression of β-catenin is more often detected in patients with FIGO stage III and IV than in those with stage I, and II (P=0.003). No significant relationship was found between expression of β-catenin and pathological grade (P=0.817). Positive expression of β-catenin related to lower survival rate (P=0.034). The expression of cyclinD1 had no relationship with FIGO stage (P=0.829). Overexpression of cyclinD1 was positively to pathological grade (P=0.017) and survival rate (P=0.009). There is a significantly positive relationship between expression of β-catenin and cyclinD1 (P=0.014). No statistical significance was found between expression of β-catenin and cyclinD1 and other pathological parameters. *Conclusions:* Expression of β-catenin and cyclinD1 may be used as predict markers for poor prognosis.

Keywords: β-catenin, cyclinD1, ovarian serous carcinoma, prognosis

Introduction

Ovarian cancer is the fifth most common cancer among women in developed countries and the leading cause of death in women with gynecologic malignancies, with a death toll up to 13850 in the USA by 2010 [1]. The incidence of ovarian cancer in 2009 in China, according to data from Chinese Cancer Registry Annual Report, is 7.95/10^5, which ranks 10th in most common cancers in female. Ovarian cancer ranks at 9th in the leading causes of cancer death in urban areas of female in 2009. Though ovarian cancer is not the most common cancer in female in China, it is the common causes of cancer death in female because that its symptoms are vague, non-specific, and generally appear in the advanced stages of the disease. All over the world, improvement in surgical management and advances in cytotoxic therapy have been accomplished in the past decades, however, the overall 5-year survival rate for women with advanced disease can be low as 13% [2]. The majority of cases are related with recurrence and chemoresistance. Although several prognostic markers have been evaluated, tumor stages, residual diseases after surgery, histological types, and tumor grades are the most important prognostic factors, which are still insufficient to identify high-risk patients. Therefore, specific prognostic markers for ovarian cancer progression are still warranted. Elucidation of the molecular mechanisms involved in the pathogenesis of ovarian cancer may improve survival rates though better therapy as well.

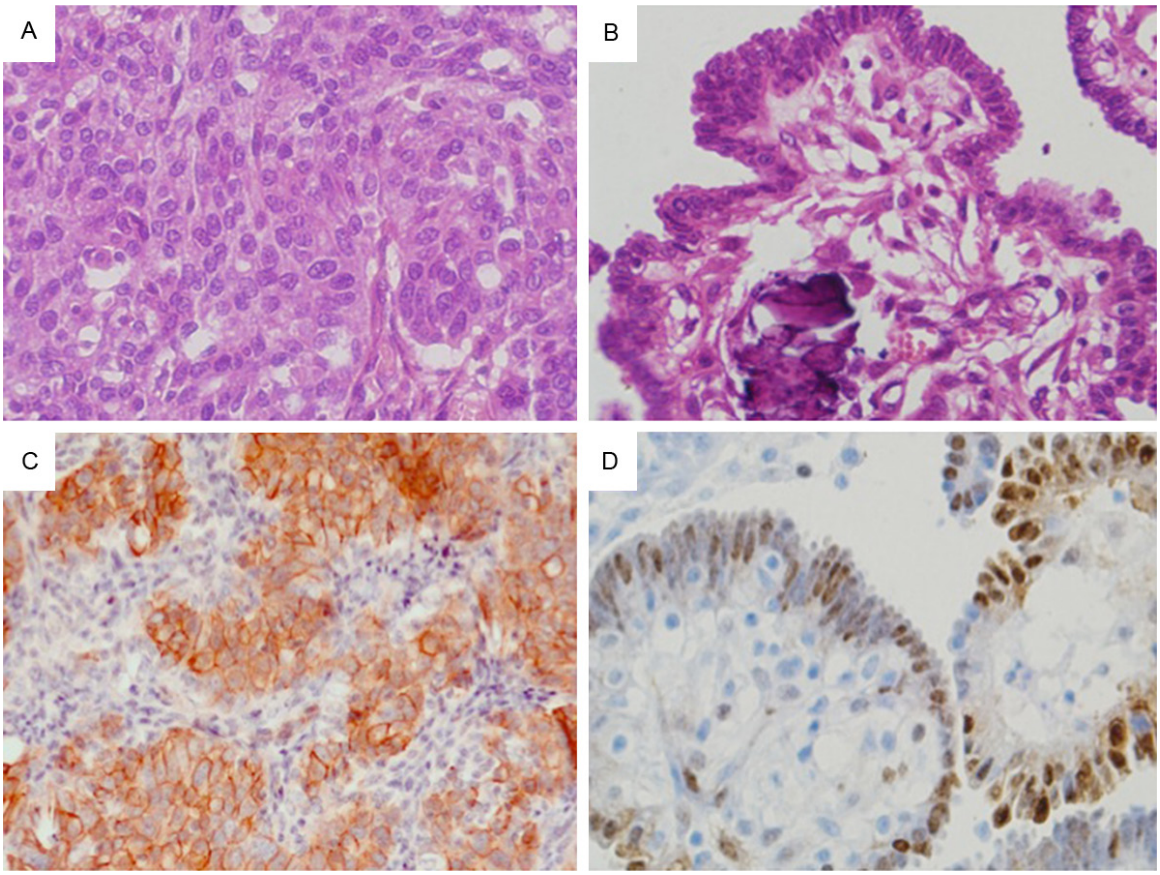

Figure 1. A and B: Typical HE staining of high-grade and low-grade of ovarian serous carcinoma. C and D: Immunohistochemical staining of β-catenin and cyclinD1.

The Wnt pathway normally plays a role in growth and differentiation. A key downstream component of the Wnt pathway, the protein β-catenin, initially discovered as a component of cell-cell adhesive junctions and is regulated by its association with adenomatous polyposis coli (APC) and glycogen synthase kinase-3beta (GSK3-beta). In colon cancer, studies have shown that a mutation in either the APC or the β-catenin gene can result in accumulation of the β-catenin complex in the cytoplasm and nucleus. This results in increased transcriptional activity with downstream effects of cyclinD1, c-myc, and peroxisome proliferator-activated receptors. In the normal cell, degradation of β-catenin through the ubiquitin pathway is facilitated by phosphorylation. Mutational changes to the complex will lead to an increased free pool of β-catenin in the cytoplasm. Apart from being important for cell-cell adhesion, β-catenin also binds to the transcription factor T-cell factor/lymphocyte enhancer factor (Tcf/Lef) c-myc, E-cadherin and cyclin D1. CyclinD1 is a member of the G1 cyclins and is a major positive regulator of the G1 restriction point. CyclinD1 is an essential sensor and activator of cell cycle initiation and progression. Overexpression of cyclinD1 renders the growth of normal cells less dependent on growth factors and accelerates passage through the G1 phase of the cell cycle, suggesting that increased expression may lead to the loss of normal regulatory constraints and confer a growth advantage. Overexpression of cyclinD1 is linked to various human cancers, including esophageal cancer [3], breast cancer [4], and pancreatic cancer [5].

As far as we are aware, just a few previous studies have analyzed the prognostic significance of β-catenin and cyclinD1 expression in ovarian serous cancer. The association of β-catenin combining cyclinD1 expression with clinicopathologic parameters and survival is not well known. In the present study, we detected expression of β-catenin and cyclinD1 protein in

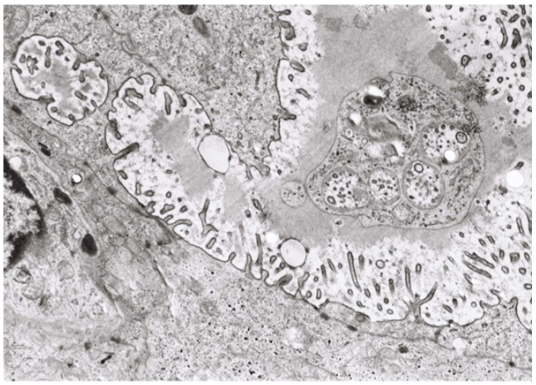

Figure 2. Ultrastructural examination showed serous carcinoma cells. They had slender, straight microvilli of variable length present on the covering apical surfaces of the luminal and papillary. There were abundant well-formed desmosomes and glycocalix.

a set of ovarian serous carcinoma samples by immunohistochemistry. The relationship between β-catenin and cyclinD1 expression and clinical stages, pathologic grade and survival was analyzed.

Material and methods

Patients and clinicopathologic variables

Archival paraffin-embedded tissue blocks from 60 patients (range 22-79, mean 51 years) with serous cancer of ovary were obtained from Jinling Hospital, Nanjing, China, between the years 1997 and 2006. All hematoxylin-and eosin-stained slides were re-reviewed by a gynecological pathologist to verify the diagnosis, histological grade, and stages. Women with other histological subtypes were excluded from present study. Pathological stage and histological subtype were determined for each surgical specimen according to 2002 International Federation of Gynecology and Obstetrics (FIGO) criteria, and Pathology and Genetics Tumors of the Breast and Female Genital Organs (World Health Organization, WHO 2003). A two-tier (low-grade and high-grade) system was used to define the differentiation of ovarian serous carcinoma. Papillary pattern is of representative lwo-grade differentiation, whereas solid pattern is of representative high-grade differentiation of ovarian serous carcinoma (**Figure 1A**, **1B**). Patient data were obtained from hospital tumor registry and chart review. All cases of recurrence had radiographic evidence of disease or biopsy proven progression of disease. Ethical approval was obtained from Jinling Hospital Ethics Board. Follow-up ranged from 2.5-141 months, and average was 61.7 months. The records of patients who were alive at follow-up or who did not die of disease were considered to be censored. To histologically differentiate the high-grade ovarian serous carcinoma from poor differentiated endometrioid adenocarcinoma and ovarian mucinous carcinoma, transmission electron microscopy (TEM) images were used. Ultrastructural examination showed serous carcinoma cells. They had slender, straight microvilli of variable length present on the covering apical surfaces of the luminal and papillary. There were abundant well-formed desmosomes and glycocalix (**Figure 2**).

Immunohistochemistry

Sections from surgical specimens had been fixed in 10% formalin and embedded in paraffin and they were used for immunohistochemical staining according to a standard method. Briefly, each 4-μm tissue section was deparaffinized and rehydrated. After rehydration through a graded ethanol series, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval, then cooled to 30°C and washed with phosphate-buffered saline (PBS, pH 7.3). After endogenous peroxidase had been quenched with aqueous 3% H_2O_2 for 10 minutes and washed with PBS, the sections were incubated at 4°C overnight with a β-catenin monoclonal mouse antibody (Dako, Denmark A/S Produktionsvej 42 DK-2600 Glostrup) and cyclinD1 monoclonal mouse antibody (Neomarkers, USA) at a 1:50 dilution in antibody diluent solution (Zymed, Invitrogen) and then washed with PBS. Next, the sections were incubated with secondary antibody (Dako REAL EnVision Detection System, Dako, UK) for 30 min at room temperature. Color development was performed with 3, 3'-diaminobenzidine (DAB). Nuclei were lightly counterstained with hematoxylin. Two pathologists independently assessed the immunostained slides. Any difference in immunohistochemical scores was resolved by a consensus.

The β-catenin staining of the membrane, cytoplasm, and nucleus was evaluated as described by C.M. Lee et al [6]. The staining of cytoplasm and nucleus was considered as positive staining of β-catenin. Immunohistochemical staining

Table 1. Association of beta-catenin and cyclinD1 with FIGO stage in ovarian serous carcinoma

FIGO stage	No.	beta-catenin expression			*P* Value	cyclinD1 expression			*P* Value
		-	+	++		-	+	++	
I	7	4	1	2		3	4	0	
II	7	3	1	3		2	2	3	
III	39	6	17	16	0.003	12	17	10	0.829
IV	7	0	2	5		3	2	2	

Table 2. Association of beta-catenin and cyclinD1 with pathologic grade in ovarian serous carcinoma

grade	No.	beta-catenin expression			*P* Value	cyclinD1 expression			*P* Value
		-	+	++		-	+	++	
low	26	6	8	12	0.817	13	8	5	0.017
high	34	6	13	14		7	17	10	

of cancer cells was semi-quantitatively assessed according to the percentage of positive cells. Percentage of cells that were stained in each section was numbered as follows: "0" denoted <5% staining, "1" denoted 5-50%, and "2" denoted >50% [6]. The cyclinD1 staining was evaluated and referred to previous reported method [7]. Briefly, "-": <10% of cells stained; "+": 10-50% of cells stained; "++": >50% of cells stained. HER2 positive is defined as membrane staining. 0: No staining is observed in invasive tumor cells. 1+: Weak, incomplete membrane staining in any proportion of invasive tumor cells, or weak, complete membrane staining in less than 10% of cells. 2+: Complete membrane staining that is non-uniform or weak but with obvious circumferential distribution in at least 10% of cells, or intense complete membrane staining in 30% or less of tumor cells. 3+: Uniform intense membrane staining of more than 30% of tumor cells. For each case, a corresponding section was incubated with nonimmune mouse serum as a negative control.

Fluorescence in situ hybridization (FISH)

Probes specific for centromeres of chromosomes 17 and for the HER2 locus were provided by GP Medical Technologies, Ltd, Beijing, China. FISH was carried out according to the manufacture's instruction. Briefly, 4-μm tissue section was deparaffinized and rehydrated. Following a 60 min of protease digestion and denaturation, tissue sections were hybridized for 16 hours at 37°C with probes. After hybridization, slides were washed and applied to a DAPI (4', 6-diamidino-2-phenylindole) counterstain. The scoring system proposed by Vysis was used to quantify HER2 signals. At least 30 nonoverlapping, interphase tumor cell nuclei were evaluated. In each nucleus, the number of HER2 and chromosome 17 centromere signals was counted. The HER2/CEP17 ratio was calculated.

Statistical analysis

The statistical significance of intergroup differences was evaluated by a chi-square test. Kaplan-Meier survival curve was calculated using breast cancer-related death (overall survival) as the endpoints. All statistical analyses were performed using SPSS software (SPSS 16.0, Chicago, IL). A two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Clinicopathologic characterization

The mean patients age at the time of diagnosis was 52 years (range 22-79 years). Lymph node sampling or dissection found that 36 patients (60%) having lymph node metastasis. Seven (12%) patients were diagnosed in FIGO stage I, 7 (12%) patients in stage II, 39 (65%) patients had FIGO stage III and 7 patients (12%) presented with metastatic disease (FIGO IV). Two-tier grade system was used in our ovarian serous carcinomas. Low-grade (n=26) and high-grade (n=34) was grouped.

β-catenin and cyclinD1 protein expression in serous ovarian carcinoma

The β-catenin protein was localized in the membrane, cytoplasm, and nucleus of ovarian

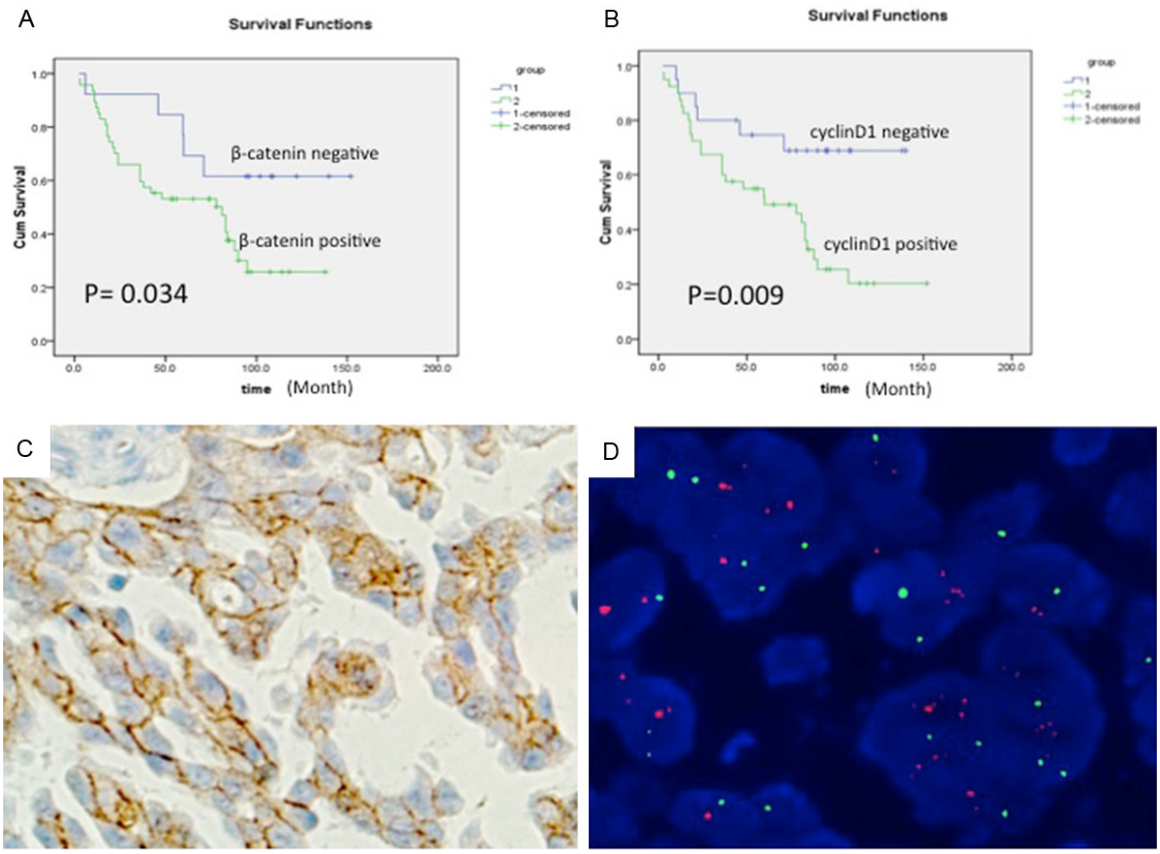

Figure 3. Kaplan-Meier survival curve showed significant effect of positive expression of β-catenin in ovarian serous carcinoma (A). Kaplan-Meier survival curve showed significant effect of positive expression of cyclinD1 in ovarian serous carcinoma (B). The membrane positive staining of HER2 in ovarian serous carcinoma (C). FISH analysis of HER2 amplification in ovarian serous carcinoma (D).

serous carcinoma cells (**Figure 1C**). The predominantly positive immunostaining was localized in both of membrane and cytoplasm. The unique membrane staining of β-catenin in ovarian serous carcinoma samples was not found. The nuclear expression of cyclinD1 was immunostained in 67% of patients with serous ovarian carcinoma (**Figure 1D**). The staining level of β-catenin and cyclinD1 protein was varied inter- and intra-samples.

Association of β-catenin and cyclinD1 expression with FIGO stage

Table 1 shows the association between EphB1 and cyclinD1 expression and FIGO stage. Overexpression of β-catenin is more often detected in ovarian serous carcinomas with FIGO III and IV (87%) than in FIGO I and II (50%, *P*=0.003). No relationship was found between expression of cyclinD1 and FIGO stage (*P*=0.829).

Relationship between expression of β-catenin and cyclinD1 and pathologic grade

The positive staining of β-catenin was detected in 77% and 79% of low-grade and high-grade serous ovarian carcinomas (*P*=0.817). The cyclinD1 was detected in 50% of low-grade and 79% of high-grade serous ovarian carcinomas (*P*=0.017). The results were listed in **Table 2**.

Overexpression of β-catenin and cyclinD1 protein is correlated to a poor overall survival

Fifty-five cases stained for β-catenin and cyclinD1 had follow-up data for survival and were available for assessment. At the end of the follow-up period, 34 (62%) patients were dead of their ovarian carcinoma. Overexpression of β-catenin and cyclinD1 was significantly associated with overall poor survival (**Figure 3A**, *P*=0.034 and **Figure 3B**, *P*=0.009).

Expression and amplification of HER2

We examined the HER2 protein expression and amplification in 51 cases of ovarian serous carcinoma by using immunohistochemical staining and fluorescence *in situ* hybridization (FISH). Three out of 51 samples (5.8%) were detected 3+ membrane staining of HER2 (**Figure 3C**). Nine out of 51 samples showed 1+ or 2+ positive staining of HER2. Only one sample was detected HER2 amplification by FISH, and the HER2/CEP17 ratio was 2.1:1 (**Figure 3D**).

Discussion

β-catenin has been previously associated with oncogenic activity in human cancers. Increasing number of data show that β-catenin has been involved in carcinogenesis, progression and metastasis of ovarian carcinoma [8-13]. The role of β-catenin protein is diverse and depends on its cellular localization. E-cadherin plays an important role in the formation of cell junctions and is a key regulator of the differentiated epithelial cell phenotype. E-cadherin forms a complex with a group of peripheral membrane linkage proteins including β-catenin. Down-regulation of cell membrane expression of β-catenin has been associated with low histologic differentiation of carcinomas, increased risk of local invasion of the tumor, metastatic disease, and poor survival in cancer patients. Another important molecular mechanism including β-catenin that has been implicated in tumorigenesis is the Wnt pathway. β-catenin, as the key downstream component of the Wnt pathway, is regulated by its association with adenomatous polyposis coli (APC) and glycogen synthase kinase-3β in colon cancer. Activated β-catenin in the Wnt pathway is accumulated in the cytoplasm and nucleus. In the present study, the β-catenin protein was predominantly localized in the membrane and cytoplasm of ovarian serous carcinoma cells. Only few samples showed a nucleus staining of β-catenin protein (five out of 60 ovarian serous carcinomas). The expression of β-catenin protein in ovarian serous carcinoma samples showed inter- and intra-heterogeneity. We found that the loss of expression of β-catenin protein in cell membrane is often associated with high-grade carcinoma (data not show). In this study, we focus on the relationship between expression of β-catenin protein both in cytoplasm and nucleus and clinicopathologic parameters. Considering rare cases showed nuclear staining, we did not analyze the relationship between nuclear localization of β-catenin protein and clinicopathologic parameters. Our results show that expression of β-catenin protein is positively associated with advanced FIGO stage, but not with tumor grade. Positive expression of β-catenin was significantly associated with overall poor survival. Lu et al reported that β-catenin nuclear localization is associated with grade and median survival of patients with ovarian serous carcinoma [6]. They found that thirteen of 105 serous tumors (12.3%) demonstrated nuclear staining of β-catenin. Nuclear staining was found in 23% of high-grade carcinoma and 2.1% in low-grade carcinoma (P=0.006). Their data indicated the positive staining of β-catenin in nucleus, cytoplasm or in membrane is independent of tumor grade. However, our data showed that predominantly positive immunostaining was localized in both of membrane and cytoplasm. We did not find that positive staining of β-catenin was only in cell membrane or nucleus in ovarian serous carcinoma samples. Lu et al reported that no statistically significant relationship was found between membrane staining or cytoplasmic staining of β-catenin and tumor grade.

CyclinD1 plays a critical role in regulation of cell cycle progression. Lin et al identified a perfect T cell factor 4 (Tcf4)-binding site (CTTGATC) located between nucleotides -80 and -73 in the promoter region of cyclinD1 and suggesting the potential involvement of the β-catenin/Tcf4 pathway in the regulation of cyclinD1 expression [14]. By using breast cancer cell lines they confirmed the Tcf4 interaction with Cyclin D promoter and showed involvement of β-catenin/Tcf4 pathway in Cyclin D promoter activation. Tanaka et al investigated the association between the expression of cyclinD1, pRb, p16, p53, $p27^{Kipl}$, $p21^{Wafl/Cipl}$ and cylinE and clinicopathologic parameters. They found overexpression of cyclinD1 was positively correlated with reduced progression-free survival, overall survival and first-line chemosensitivity [15]. In the present study, the expression of cyclinD1 was assessed by immunostaining in 67% of patients with serious ovarian carcinoma. The positive expression of cyclinD1 was more often detected in high-grade serous ovarian carcinomas (P=0.017), but not associated with FIGO stage (P=0.829). There is a significantly positive rela-

tionship between expression of β-catenin and cyclinD1 (P=0.014). Overexpression of cyclinD1 was significantly associated with poor overall survival (P=0.009).

HER2 is a member of the epidermal growth factor family of tyrosine kinase receptors involved in cellular proliferation and tumor cell metastasis. Amplification or overexpression of HER2 has been reported in up to 30% of breast cancer. Breast cancer patients with HER2 positive or amplification have a poor prognosis. HER2 overexpression and amplification has been reported in ovarian carcinomas, but the exact percentage of HER2 positive tumors is varied in the literature [16-23]. In the present study, we examined the HER2 protein expression and amplification in 51 cases of ovarian serous carcinoma by FISH. Three out of 51 samples (5.8%) were detected 3+ membrane-staining of HER2. Nine out of 51 samples showed 1+ or 2+ positive staining of HER2. Only one sample was detected HER2 amplification by FISH. Our data show that the percentage of amplification or over expression of HER2 in ovarian serous carcinoma is lower than that reported in ovarian mucinous carcinoma [16, 24].

In summary, overexpression of β-catenin is more often detected in patients with advanced FIGO stage. No significant relationship was found between expression of β-catenin and pathological grade. Positive expression of β-catenin related to lower survival rate. Overexpression of cyclinD1 was positively related to pathological grade and survival rate, but not to FIGO stage. There is a significantly positive relationship between expression of β-catenin and cyclinD1. No statistical significance was found between expression of β-catenin and cyclinD1 and other pathological parameters. In conclusion, expression of β-catenin and cyclinD1 may be used as a marker for poor prognosis.

Acknowledgements

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Disclosure of conflict of interest

The authors declare that they have no conflict interest.

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