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CD24, COX-2, and p53 in epithelial ovarian cancer and its clinical significance

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1. ABSTRACT

Epithelial ovarian cancer (EOC) prognosis is associated with International Federation of Gynecology and Obstetrics (FIGO) staging, cancer cell classification, patient age, and residual tumor size. However, the molecular markers for predicting EOC prognosis remain to be explored. In this study, we investigated the expression of CD24, COX-2, and p53 in EOC, and their relationships to clinical prognosis. We found that the expression of CD24 was detected in the cell membrane in 90.6% (58/64) of EOC cases and in the cytoplasm in 54.7% (35/64) of EOC cases; 78.6% (11/14) cases of borderline tumors had CD24-positive staining in the cell membrane. All 9 cases of benign tumors were negative for CD24 staining. Expression of CD24 correlated with the nuclear expression of p53, but not with the expression of COX-2. Overexpression of CD24 is an independent factor associated with tumor metastasis, a low survival rate, and a short survival time. Our results suggest that CD24 may be a valuable molecular marker for predicting prognoses of patients with EOC.

2. INTRODUCTION

Ovarian cancer is the leading cause of death from gynecologic malignant tumors. Approximately 21,600 new ovarian cancer cases were diagnosed in the United States in 2008 (1). The therapeutic effects of ovarian cancer treatment are still unclear because more than 70% of diagnosed patients are already in the advanced stages of disease. The 5-year survival rate is still approximately 30%, although radio therapy and chemotherapy technologies for cancer therapy have improved in recent years. Traditionally, EOC prognosis has been determined according to FIGO staging, cell classification, patient age, and residual tumor size (2). However, physicians and pathologists are still trying to determine the best prognostic indicator to guide patient treatment (3-5). The application of molecular biological technologies for exploring valuable prognostic indicators of ovarian cancer will have significant implications.

CD24 is a P-selectin ligand for human tumor tissue, also known as a heat-stable antigen (HAS). It is a glycoprotein that is expressed in neutrophils, B cells, immature thymocytes, and red blood cells. CD24 was initially detected in blood cancers such as leukemia and lymphoma, and was later found to be overexpressed in solid tumors, such as small cell lung cancer and ovarian cancer (6-8). CD24 is expressed on the cancer cell surface, and is a kind of transfer-promoting factor, which can bind with P-selectin on platelets or vascular endothelial cells, and in this way, promote cancer metastasis (9). If CD24 is overexpressed in ovarian cancer cells, patients have a higher degree of malignancy with poorer prognoses and shorter survival times. This indicates that CD24 plays a critical role in cancer development and progression. Expression of CD24 has an instructive value for a patient's postoperative prognosis.

Cyclooxygenase-2 (COX-2) is a proinflammatory enzyme that is mainly expressed in neovascular endothelial cells, macrophages, and tumor cells. COX-2 catalytically produces a variety of inflammatory factors including prostaglandins (PG), which are then involved in the pathological process of inflammation and tumor angiogenesis. Seo et al. (10) observed overexpression of COX-2 in EOC, suggesting strong cancer invasion and very short survival periods for EOC patients. Overexpression of COX-2 is an independent prognostic factor.

The tumor suppressor gene *p53* initiates tumors when it is mutated or deleted. The mutated *p53* gene is present in approximately 30–80% of EOC cases. The accumulation of mutant p53 protein in EOC is related to primary tumor volume. The mechanism of poor prognosis in EOC patients with *p53* mutations provides an effective clinical target (11).

In this study, we detected expression of CD24, COX-2, and p53 in EOC using immunohistochemical staining, and explored the relationship between CD24 expression and EOC patient prognosis.

3. MATERIALS AND METHODS

3.1. Patient clinical data

This study was approved by Tongji University (Shanghai 200040, China). A total of 87 patients who underwent surgery at Shanghai First Maternity and Infant Hospital between January 1998 and January 2003 were recruited for this study. Pathologic patient diagnoses were made according to WHO criteria. Among the 87 patients, 9 had benign cystadenomas, 14 had borderline tumors, and 64 had EOCs, which were used for immunohistochemical detection of CD24, COX-2, and p53 expression. The average age of patients was 49.5 years (range, 22–75 years). In terms of FIGO staging, 31 cases were stage I (48.4%), 6 cases were stage II (9.4%), 24 cases were stage III (37.5%), and 3 cases were stage IV (4.7%). In terms of pathology classification, 30 cases were grade I (46.7%), 16 cases were grade II (25%), and 18 cases were grade III (28.1%). None of the patients received chemotherapy before surgery, and platinum was typically used for postoperative chemotherapy. Patients in stage I received 6 cycles of postoperative chemotherapy; patients in stages II–IV received 8 cycles of chemotherapy. Cell grading and histopathological type were re-verified by 2 highly experienced pathologists.

Overall survival (OS) was calculated from the time of the first surgery until the death date or final inspection date. Disease-free survival (DFS) refers to the interval period between the first clinical remission after treatment and tumor recurrence. Follow-up with all patients took place by telephone, letter, or outpatient appointments.

3.2. Immunohistochemistry

To determine immunohistochemistry, formalin-fixed, paraffin-embedded tissue was cut into sections. Antigen retrieval was achieved by treatment in a high-temperature pressure cooker heated for 90 s in a citrate buffer, pH 6.0. Sections were incubated in 0.5% H_2O_2 to quench endogenous peroxidase activity and then blocked with normal goat serum. The slides were then incubated with primary monoclonal anti-human antibodies against CD24 (dilution 1:20; Antibody Diagnostica Inc., USA), COX-2 (dilution 1:50; Antibody Diagnostica Inc., USA), or p53 (dilution1:20, Antibody Diagnostica Inc., USA) for 1 h at room temperature. The resulting slides were incubated in a humidity chamber for 10 min each with biotinylated secondary antibodies and streptavidin with intervening and subsequent rinses in PBS 3x for 5 min. Then, 3,3′-diaminobenzidine-tetrahydrochloride was applied as a chromogen for 5 min. Finally, sections were counterstained in hematoxylin for 1 min.

3.3. Image analysis

Positive staining was independently diagnosed by 2 pathologists. For CD24, cells with brown granules in the cell membrane, cytoplasm, or both were positive. For COX-2, cells with brown granules in the cytoplasm were positive. For p53, cells with brown granules in the nucleus were positive. The positive cells had clear cell structures, characteristic positions, and significantly better color staining than the backgrounds of positive granules. The scores of positive staining were graded using an immune response scoring (IRS) standard based on staining intensity (SI) and positive percentage (PP), IRS = SI × PP. In scoring SI, we used 0 for no positive cells, 1 for weak positivity, 2 for medium positivity, and 3 for strong positivity. In scoring PP, we used 0 for no positive cells, 1 for 1–10% positive, 2 for 11–50% positive, 3 for 51–80% positive, and 4 for > 80% positive. IRS was classified into negative (0), low expression (1-4) and strong expression (> 4) (see Figure 1)

3.4. Statistical analysis

The data were statistically analyzed using SPSS software, version 11.0. The mean sample difference was analyzed using a Student's *t*-test. The rate difference was assessed using the χ^2 test. Correlation tests for 2 variables were performed using the Spearman rank correlation test or the non-parametric test. Survival rate was depicted using the Kaplan-Meier survival curve. Differences in survival rates were assessed using the log-rank test. Prognostic

Table 1. Immunohistochemical detection of CD24 expression in EOC and its correlation to clinical pathology

Items	Cases	CD24	χ^2	p-value		
		Negative	Low	High		
Age						
≤50 yrs	36	4	22	10	0.864	0.293
>50 yrs	26	2	14	12		
Pathological type						
Clear Cell carcinoma	23	0	13	10	2.845	0.241[a]
Ovarian endometrial adenocarcinoma	18	2	8	8	3.574	0.467[b]
The others EOC	23	8	15	4		
Staging						
I + II	37	4	20	13	0.283	0.868
III + IV	27	2	16	9		
Differentiation						
I + II	46	3	27	16	1.602	0.449
III	18	3	9	6		
Residual tumor size						
≤1cm	42	4	24	14	0.059	0.971
>1cm	22	2	12	8		
Gastrocolic omentum metastasis						
No	34	4	23	7	6.127	0.047
Yes	30	2	13	15		

a: comparison between clear cell carcinoma and ovarian endometrial adenocarcinoma, b: comparison between ovarian endometrial adenocarcinoma and others.

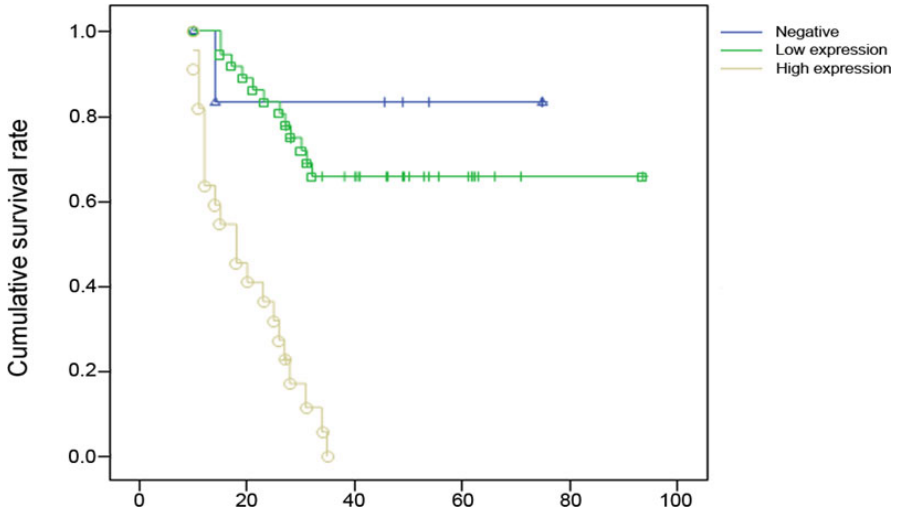

Figure 1. Expression of $CD24^+$ cell in ovarian endometrioid adenocarcinoma. A: Negative expression of $CD24^+$ cell (Original magnification: 100X). B: Low expression of CD24+ cell (identified as cytomembrane immunolocalization; Original magnification: 200X). C: Strong expression of CD24+ cell (identified as cytomembrane immunolocalization; Original magnification: 400X). D: CD24 expression was identified both as cytomembrane immunolocalization and cytoplasm immunolocalization (Original magnification: 400X).

multivariate analysis was performed using the Cox proportional hazards model.

4. RESULTS

4.1. CD24 expression in EOC cells

CD24 was expressed mainly in the cell membrane and to a lesser extent in the cytoplasm of EOC cells. Among the 64 cases (23 ovarian clear cell carcinoma, 18 ovarian endometrial adenocarcinoma, 12 ovarian mucinous adenocarcinoma, 4 ovarian serous adenocarcinoma, 3 ovarian adenosquamous carcinoma, and 4 other EOC), 58 (90.6%) exhibited positive CD24 expression on the cell membrane. Among the 58 cases, 36 (62.1%) had low expression, 22 (37.9%) had high expression, and 6 (9.4%) were negative for CD24 staining. For cytoplasmic staining, 35 cases (54.7%) were positive and 29 cases (45.3%) were negative. All 9 cases of benign tumors (serous or mucinous cystadenoma) showed negative staining. Among the 14 cases of borderline tumors, 11 (78.6%) showed positive staining in the cell membrane, one of which had strong CD24 expression in both the cell membrane and the cytoplasm. This tumor was in stage I and had normal follow-up (Figure 1).

In comparing CD24 expression in EOC according to age, disease stage, cell differentiation, pathological type, residual tumor size, and presence of metastasis on the gastrocolic omentum (Table 1), we found that CD24-positive staining significantly correlated with metastasis to

Table 2. The effects of CD24 expression at the cell membrane on the survival rate of EOC patients

Factors	Single-factor analysis	Multi-factor analysis		
	p-value	Risk ratio	95% CI	p-value
Age (≤ 50 Vs >50 years old)	0.5935	0.397	0.270-1.280	0.181
Differentiation (I + II vs III)	0.0074	0.494	0.348-2.418	0.862
Stages (I + II vs III – IV)	0.0002	0.783	0.415-8.912	0.404
Pathological type (clear cell carcinoma vs ovarian endometrioid adenocarcinoma)	0.8230	0.675	1.127-15.881	0.033
Pathological type (ovarian endometrioid adenocarcinoma vs the other EOC)	0.8230	0.450	1.087-6.352	0.032
Residual tumor size (≤1cm vs >1cm)	0.0002	0.440	0.772-4.334	0.170
Gastrocolic omentum metastasis (no vs yes)	0.0458	0.581	0.204-1.997	0.441
CD24 (IRS 0.1-4 vs > 4)	0.0000	0.512	3.014-22.385	0.000

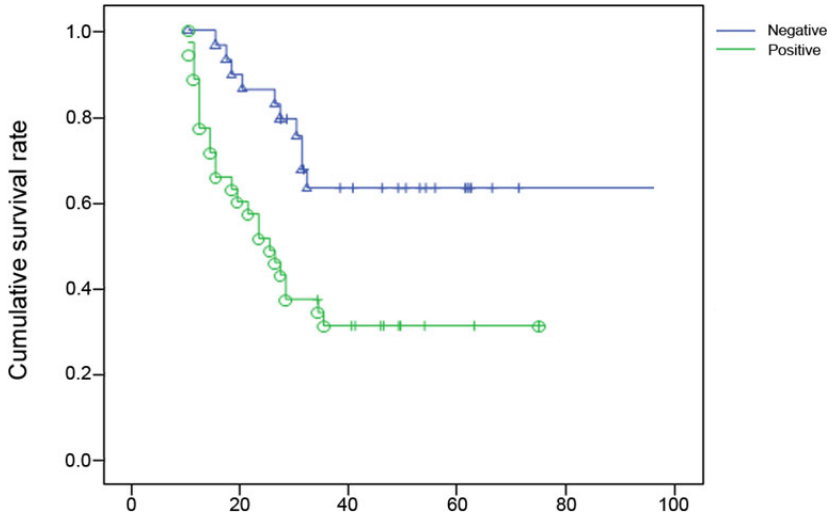

Figure 2. The expression of CD24 at the membrane expression affected the overall survival rate of EOC patients in Kaplan-Meier graph.

the gastrocolic omentum (p = 0.047); however, CD24 expression did not correlate with other prognostic factors.

4.2. Correlation of CD24 expression with COX-2 and p53 in EOC

Thirty-four cases (53.1%) tested positive for COX-2 expression, including 8 cases with low expression and 26 cases with high expression. Thirty cases (46.9%) had COX-2 negative expression. Nuclear expression of p53 was detected in 29 cases (45.3%), including 18 cases with low expression and 11 cases with high expression; 35 cases (54.7%) tested negative for p53 expression. IRS scores and the Spearman rank correlation test showed that expressions of CD24 and COX-2 did not correlate (p = 0.127, r = 0.193), expressions of CD24 and p53 correlated ($p < 0.05$, r = 0.563), and expressions of COX-2 and p53 did not correlate with one another (p = 0.164, r = 0.176).

4.3. The relationship between CD24 expression and the survival rate of EOC patients

Follow-up with all 64 EOC patients continued until January 31, 2005. Of the patients, 34 died, 28 survived with no tumors, and 2 survived with tumors. The 1-year, 2-year, and 3-year survival rates of EOC patients were 84.4%, 65.6%, and 46.9%, respectively.

Based on the single factor log-rank test, EOC patients with poor differentiation (p = 0.0074), later FIGO stages (p = 0.0002), larger residual tumor sizes (p = 0.0002), metastasis to the gastrocolic omentum (p = 0.0458), and high CD24 expression (p = 0.000) experienced shorter survival periods.

The 3-year survival rates for patients with negative, low, and high CD24 expression were 83.3%, 66.7%, and 4.6%, respectively. Their total average survival time was 35.1 months, and differences in 3-year survival rates were significant based on the single factor log-rank test. The average survival times for patients with CD24-negative, low, and high expression were 65 months, 70 months, and 20 months, respectively; age, pathologic types, and expressions of COX-2 and p53 were not correlated with prognosis. The survival rates for patients with CD24-negative, low, or high expression were significantly different (p = 0.0001) based on the Kaplan-Meier survival curve (Figure 2). Prognostic multivariate analysis indicated that CD24 (p = 0.0001) and pathological type (p = 0.032/0.033) were independent factors that affected patient survival rate and prognosis (Table 2).

The average survival time for EOC patients with CD24 expression in the cytoplasm was 25 months; for patients without CD24 expression, the average survival time was 36 months. The related Kaplan-Meier survival curve is presented in Figure 3. Prognostic multivariate analysis indicated that CD24 (p = 0.005) is an independent factor associated with poor prognosis for EOC patients (Table 3).

Table 3. The effects of CD24 expression in the cytoplasm on the survival rate of EOC patients

Factors	**Single-factor analysis**	**Multi-factor analysis**		
	p-value	**Risk ratio**	**95% CI**	**p-value**
Age (≤ 50 Vs >50 years old)	0.5935	0.392	0.522-2.424	0.764
Differentiation (I + II vs III)	0.0074	0.484	0.337-2.247	0.774
Stages (I + II vs III – IV)	0.0002	0.677	0.874-12.399	0.078
Pathological type (clear cell carcinoma vs ovarian endometrioid adenocarcinoma)	0.8230	0.576	0.340-3.244	0.932
Pathological type (ovarian endometrioid adenocarcinoma vs the other EOC except clear cell carcinoma)	0.8230	0.384	0.655-2.946	0.392
Residual tumor size (≤1cm vs >1cm)	0.0002	0.434	0.643-3.530	0.346
Gastrocolic omentum metastasis (no vs yes)	0.0458	0.610	0.303-3.320	0.995
CD24 in the cytoplasm (positive vs negative)	0.0033	0.428	1.429-7.646	0.005

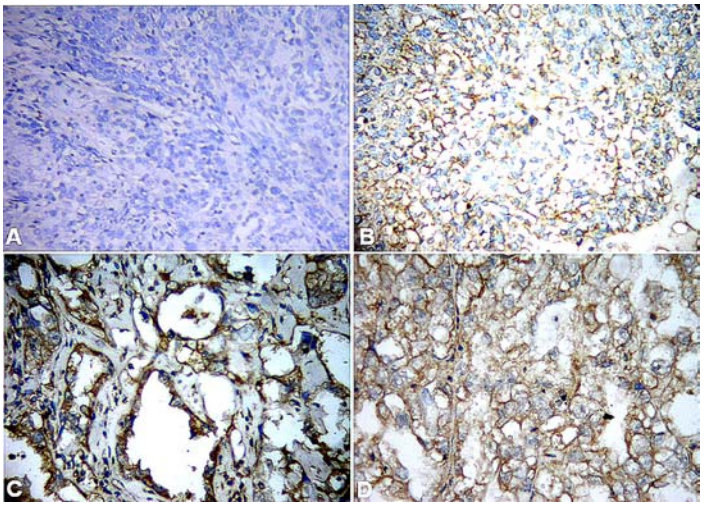

Figure 3. CD24 expression in the cytoplasm of ovarian cancer affected the overall survival rate of EOC patients in the Kaplan-Meier graph.

4.4. Factors that affect tumor-free survival rate

Of the 64 EOC patients, 59 were in complete remission after surgery, while 36 patients (56.2%) exhibited recurrence. The average tumor-free survival period was 33.6 months (range, 4–93.4 months). The 1-year, 2-year, and 3-year tumor-free survival rates were 84.4%, 65.6%, and 48.9%, respectively. Based on survival analysis, CD24 expression had the same effect on tumor-free survival rate for EOC patients (p = 0.000 in both univariate and multivariate analyses), indicating that pathological types affect EOC tumor-free survival rate (p = 0.030/0.030).

Survival rate analysis indicated that CD24 expression in the cytoplasm also affected the tumor-free survival rate (p = 0.0043 in univariate analysis, and p = 0.007 in multivariate analysis). In multivariate analysis, FIGO stage affected the tumor-free survival rate for EOC patients (p = 0.037).

5. DISCUSSION

The human *CD24* gene, with a total of 306 base pairs, is located on chromosome 20 at the q13 position. The CD24 protein contains 27 amino acids and a wide range of glycosylation sites, anchored via a glycosylphosphatidylinositol (GPI) link to the cell membrane (12, 13). The CD24 protein is *O*-glycosylated at its threonine and tyrosine residues. Researchers have hypothesized that CD24 might function through cell-cell and cell-extracellular matrix interactions, and may bind the P-selectin to platelets or endothelial cells, facilitating tumor metastasis (9). In this study, 90.6% of EOC cases were CD24-positive on the cell membrane and 35 of 64 cases (54.7%) were CD24-positive in the cytoplasm, whereas all benign tumors were CD24-negative. Of the 14 borderline tumors, 11 cases (78.6%) were CD24-positive at the cell membrane and 1 case was strongly CD24-positive in the cytoplasm. This phase I borderline tumor patient was normal at follow-up. The 3-year survival rates for EOC patients with CD24-negative, low, and high expression were 83.33%, 66.67%, and 4.55%, respectively. The differences in 3-year survival rates were statistically significant according to single-factor analysis. The results indicate that the overexpression of CD24 is associated with tumor metastasis, a high recurrence rate, and a short survival time; thus, CD24 may play a role in promoting tumor metastasis (14).

The mutant p53 protein accumulates in tumors, leading to a gradual increase in tumor size. COX-2 is a proinflammatory enzyme involved in the pathological process of angiogenesis and tumorigenesis. CD24 on the surface of tumor cells enhances adhesion with vascular

endothelial cells and platelets to promote tumor metastasis (15).

COX-2, p53, and CD24 are involved in the different stages of tumorigenesis. Huang et al. (16) found that p53 could regulate CD24 expression. In this study, we also found that the expression levels of CD24 and p53 correlated with one another ($p < 0.05$, $r = 0.563$). However, levels of CD24 and COX-2 did not correlate with each other ($p = 0.127$, $r = 0.193$). These findings may indicate that COX-2 is involved in tumor initiation, while the expression of p53 is related to a certain stage of tumor development, and the expression of CD24 is related to tumor metastasis (15). The beginning of tumor metastasis occurs after p53 expression is observed, at which point CD24 promotes and advances tumor metastasis. Whether *p53* is the regulatory gene upstream of *CD24* requires further study. In this report, we showed that the levels of CD24 and p53 expression are correlated. p53 might play an important role in the expression of CD24. Two EOC patients (bilateral ovarian clear cell carcinoma [stage III−C] and right-side ovarian clear cell carcinoma [stage I-C]) tested positive for p53, CD24, and COX-2. One patient died 10 months after surgery, and another patient died 28 months after surgery. This indicates that patients who test positive for p53, CD24, and COX-2 might have poor prognoses and shorter survival periods (8, 10, 11).

CD24 was expressed in the cell membrane and/or cytoplasm in ovarian borderline tumors. Choi et al. (17) found that approximately 26.4% of ovarian borderline tumors were CD24-positive in the cytoplasm, and borderline tumors demonstrated aggressive invasion and large gastrocolic omentum metastasis, suggesting tumor recurrence. Kristiansen et al. (18) examined 8 borderline tumor specimens, 84% of which were CD24-positive at the cell membrane. CD24 was positive in the cytoplasm in only 1 case. Of the 14 patients with borderline tumors, 11 (78.6%) were CD24-positive in the cell membrane and 1 was strongly CD24-positive in the cytoplasm. This latter patient, consistent with the report by Kristiansen et al. (18), had a stage I borderline tumor and appeared normal at follow-up. Therefore, CD24 expression can be used to predict the prognosis of borderline tumors. Larger sample sizes than those used in this study will be necessary for further research on ovarian borderline tumors.

In recent years, many research studies have focused on the important role of CD24 expression in malignant tumor metastasis. Cancer cells with high CD24 expression might invade the vessel lumen and bind with platelets, resulting in vascular or lymphatic metastasis. Kristiansen et al. first reported the immunohistochemical detection of CD24 expression in EOC patients (8). CD24 was overexpressed in the cell membrane and in the cytoplasm of cells from EOC patients. Overexpression of CD24 was an independent prognostic factor for the treatment of ovarian cancer according to the single-factor and multivariate Cox analyses. In this study, single-factor and multivariate Cox analyses suggested that overexpression of CD24 ($p = 0.000$) was the independent factor affecting EOC patient survival rates and tumor-free survival rates ($p = 0.000$ in both univariate and multivariate analyses), which was consistent with reports by Kristiansen et al. and Surowiak et al. (19, 20). In our multi-factorial analysis, pathological type ($p = 0.032/0.033$) was the independent factor affecting overall and tumor-free survival rates for EOC patients ($p = 0.030$ and 0.030 respectively), but there was no difference based on the single-factor analysis. These results show that pathological types have a definite impact on EOC prognosis.

6. ACKNOWLEDGEMENTS

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Abbreviations: EOC: epithelial ovarian cancer, HAS: heat-stable antigen, COX-2: Cyclooxygenase-2, PG: prostaglandins, IRS: immune response scoring; SI: staining intensity, PP: positive percentage, OS: Overall survival, DFS: Disease-free survival, GPI: glycosyl phosphatidylinositol

Key Words: Epithelial ovarian cancer, CD24, COX-2, p53, Prognosis

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