·Gynecologic Oncology·

The expression and significance of osteopontin and B7-H4 in epithelial ovarian neoplasm

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[Abstract] Background and Objective: Epithelial ovarian cancer involves a number of factors. Recent studies have shown that osteopontin (OPN) is related to the occurrence and development of a variety of tumors, but few studies are on ovarian cancer. B7-H4 is a newly identified tumor marker in ovarian cancer. This study explored the expression of OPN and B7-H4 and their clinical significance in epithelial ovarian tumors. **Methods:** The expression of OPN and B7-H4 in 15 cases of normal ovarian tissue, 20 of benign ovarian tumor tissue, 20 of borderline ovarian tumor tissue, and 40 of ovarian cancer tissue were detected by immunohistochemistry, and the relationship of OPN and B7-H4 expression to clinical and pathologic features of ovarian cancer was analyzed. **Results:** The levels of expression of OPN and B7-H4 were significantly higher in ovarian cancer than in borderline and benign tumors ($P < 0.05$). The positive rates of OPN and B7-H4 were significantly higher in poorly differentiated ovarian cancer than in medium and highly differentiated ovarian cancer ($P < 0.05$), and the levels of expression were significantly lower in tissue at stages I and II of ovarian cancer than in stages III and IV ($P < 0.05$). The positive rate of OPN associated with a higher rate of lymph node metastasis ($P < 0.05$), but did not relate to age and histologic type. The positive rate of B7-H4 were significantly higher in ovarian serous carcinoma than in the mucinous carcinoma ($P < 0.05$), but did not relate to age and lymph node metastasis. **Conclusion:** The expression of OPN and B7-H4 increased in epithelial ovarian cancer, which could be referenced in the diagnosis of ovarian malignant tumors.

Key words: Osteopontin, B7-H4, ovarian carcinoma, epithelial neoplasm, immunohistochemistry

Malignant ovarian tumors are one of the three types of female malignant tumors, of which epithelial ovarian cancer is the most common one, accounting for 80%–90% of ovarian cancer. Because the ovaries are located deep within the pelvic cavity, early diagnosis of ovarian cancer is difficult. As a result, between about 70%–80% of patients with ovarian cancer are already at an advanced stage at their first visit. Ovarian cancer is also prone to spread in the pelvic cavity or the abdomen as well as metastasize to the lymph nodes. Therefore, the mortality rate of ovarian cancer is the highest in tumors of the female reproductive system. Ovarian carcinogenesis involves expression disorders of oncogenes and tumor-suppressor genes, abnormal regulation of apoptosis, cell motility and migration, and so on. However, its mechanisms are not yet fully elucidated.

Osteopontin (OPN), a multifunctionally secreted phosphorylated glycoprotein that can promote cell adhesion and migration, is believed to be a secreted protein associated with malignant transformation and correlated with the carcinogenesis, development, metastasis, and prognosis of many tumors. B7-H4, a newly discovered member of the B7 family, which can negatively regulate T-cell immune response through the inhibition of T-cell proliferation, cytokine production, and cell-cycle progression, expresses at high levels in a variety of tumor tissues. The present study detected the expression of OPN and B7-H4 in 95 normal ovarian tissues, resected specimens of epithelial neoplasm, and analyzed the relationship between them and the clinical and pathologic features.

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Materials and Methods

Patient information

General information Resected ovarian tissue and specimens of epithelial ovarian neoplasm from 95 patients were obtained at the Department of Gynecology at Shanxi Cancer Hospital between

January 2008 and January 2009. Among them, 15 cases were normal ovarian tissue (obtained from patients who underwent bilateral excisions because of a single lesion in an ovary), 20 were benign ovarian neoplasms, 20 were borderline neoplasms, and 40 cases were epithelial ovarian cancer. The ages of the patients ranged between 17 years and 76 years; the median age of patients with normal ovarian tissue was 38 years; the median age of patients with benign ovarian neoplasm was 36 years; the median age of patients with borderline neoplasm was 45 years; and the median age of patients with ovarian cancer was 48 years. A total of 57 patients were older than 48 years (61.3%), among whom had 8 benign tumors, 15 borderline tumors, and 34 malignant tumors.

Pathological information All 95 specimens were verified by histological examination. Diameters of ovarian cancer tissue ranged from 1.0 to 19.0 cm and the median diameter was 13.5 cm. A total of 25 cases (62.5%) were serous carcinoma, 15 (37.5%) mucous carcinoma, and 13 (32.5%) accompanied by lymph node metastasis. According to the International Federation of Gynecology and Obstetrics (FIGO 2000) clinical stage and pathologic grade, 5 were in Stage I, 10 were in Stage II, 20 were in Stage III, and 3 were in Stage IV. As for pathological grading, 6 were in Stage G1, 10 were in Stage G2, and 24 were in Stage G3. No patient received treatment other than surgery.

Methods

Main reagents and methods Polyclonal rabbit anti-human OPN antibodies were purchased from Fuzhou Maixim Biotechnology Co., Ltd, and monoclonal rabbit anti-human B7-H4 antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. All secondary antibody kits were purchased from Fuzhou Maixin Biotechnology Co., Ltd.

Staining EnVision methods were conducted according to the manufacturer's instructions as follows: paraffin-embedded tissues were cut into 4-μm thick serial sections, deparaffinized, and underwent antigen retrieval (depending on the situation). Endogenous peroxides were blocked in newly prepared 3% (mass fraction) H_2O_2 for 10 min. After washing with phosphate-buffered saline (PBS), the sections were incubated with the primary antibody, the secondary antibody, and a streptavidin-biotin-peroxidase liquid in proper order. The color reaction was developed by incubating the sections with diaminobenzidine (DAB) for 3–5 min. Afterward, the sections were counterstained slightly with hematoxylin. Followed by dehydration and transparent, the sections were mounted with neutral gum and observed under the microscope. All specimens were cut into two 4-μm thick slices, using a section known to be positive as the positive control and PBS instead of the primary antibody as the negative control in each staining batch. Brown-yellow granules appearing in the cytoplasm, within the nucleus, or both, were defined as positive.

Determining results OPN and B7-H4 were located mainly in the cytoplasm. Semi-quantitative analysis was performed according to the percent of positively stained cells and the extent of staining: (-) no staining; (+) < 25% positively stained cells (in yellow); (++) 25%–50% positively stained cells (in yellow or brown); (+++) > 50% positively stained cells (in brown or dark brown).

Calculation of the diagnostic efficiency The sensitivity (true positive) was detected as = A / (A + C) and the specificity (true negative) was detected = D / (D + B) of OPN and B7-H4 in ovarian cancer.

Statistical analysis

A χ^2 test or Fisher Student's *t* test were used for statistical analysis. Statistical significance was assumed when $P < 0.05$. The statistical software SPSS13.0 was used to analyze the data.

Results

The expression of OPN and B7-H4 in different ovarian tissue

The expression of OPN and B7-H4 in malignant ovarian neoplasm tissue is shown in Figure 1. The positive rates in normal ovarian tissue, benign ovarian neoplasm, and borderline and malignant ovarian neoplasms are shown in Table 1. There were no significant differences between normal ovarian tissue, benign neoplasm, and borderline neoplasm in the expression of OPN ($P > 0.05$). The expression of OPN in malignant neoplastic tissue was significantly higher than in the other three groups ($P < 0.05$). There were also no significant differences between normal ovarian tissue and benign neoplasm in the expression of B7-H4 ($P > 0.05$). The expression in the borderline neoplasm and malignant neoplasm tissue were significantly higher than the other two groups ($P < 0.05$). There were no significant differences between these two groups.

The relationship between the expression of OPN and the B7-H4 protein in epithelial ovarian cancer tissue and the clinicopathologic parameters

The expression of OPN was significantly correlated with clinical stage, histologic grade, and lymph node metastasis ($P < 0.05$). However OPN expression was not related to age or histologic type. The expression of B7-H4 was correlated with clinical stage, histologic type, and the degree of differentiation ($P < 0.05$), while it was not related to age or lymph node metastasis (Table 2).

Diagnostic efficiency of the single and combined detections of OPN and B7-H4 in epithelial ovarian cancer

The sensitivity of the single detection of OPN and B7-H4 in ovarian cancer was 75.0% and 90.0%, respectively. The specificity was 89.1% and 65.5%, respectively. The sensitivity of the combined detection increased to 95.0%, while the specificity decreased to 61.8%.

Discussion

OPN and tumors

OPN, a secreted phosphorylated glycoprotein in the extracellular matrix, was discovered by Senger *et al.*[1] in an epithelial cell line undergoing malignant transformation in 1979 and named transformation-related phosphorylated protein. In recent years, studies have shown that the overexpression of OPN

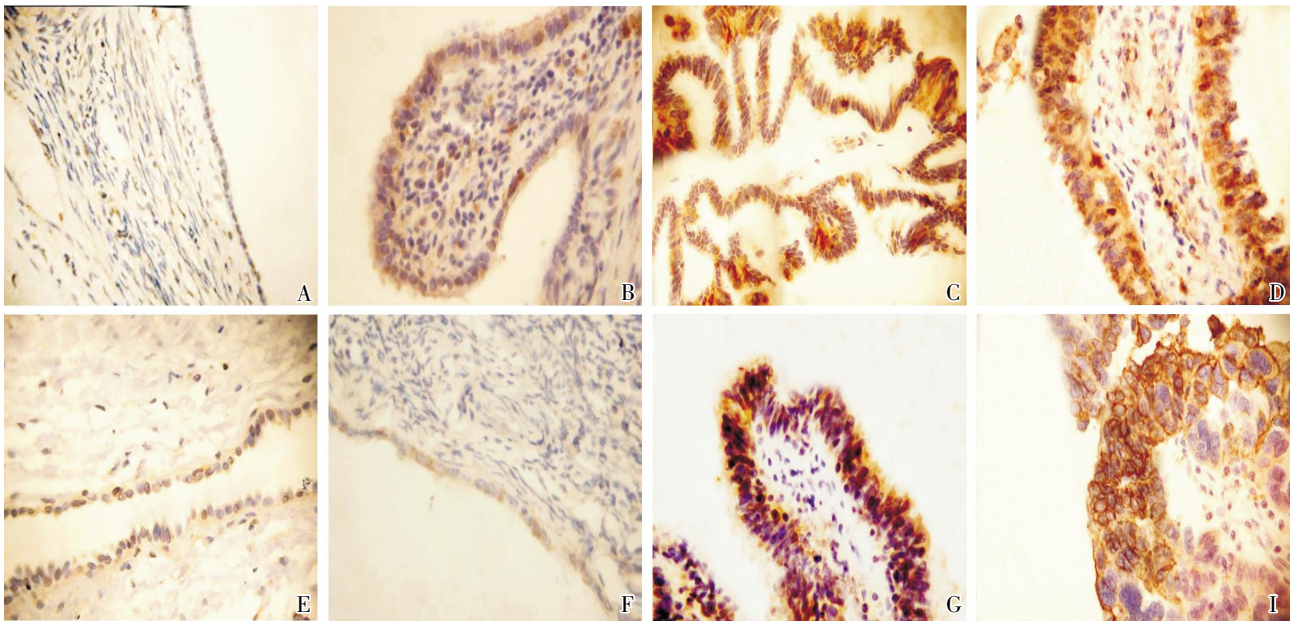

Figure 1 The expressions of osteopontin (OPN) and B7-H4 in different ovarian tissues (EnVision ×100)

A-D, the expression of osteopontin; E-I, the expression of B7-H4. A and E, nomal ovarian tumor; B and F, benign ovarian tumor; C and G, borderline ovarian tumor; D and I, ovarian cancer.

Table 1 The positive rates of OPN and B7-H4 protein expression in different ovarian tissues

Group	Patient No.	OPN		B7-H4	
		– (patient No.)	+ [patient No. (%)]	– (patient No.)	+ [patient No. (%)]
Normal ovarian tissue	15	14	1 (6.7)	13	2(10.0)
Benign ovarian tumor	20	18	2(10.0)	15	5(25.0)
Borderline ovarian tumor	20	17	3(15.0)	8	12(60.0)[b]
Malignant ovarian tumor	40	10	30(75.0)[a]	4	36(90.0)[b]

OPN, osteopontin. [a]$P<0.05$, vs. other three groups; [b]$P<0.05$, vs normal ovarian tissue and benign ovarian tumor groups.

Table 2 The relationship between the expression of OPN and B7-H4 and the clinicopathologic features of ovarian cancer patients

Characteristic	Patient No.	OPN		*P*	B7-H4		*P*
		– (patient No.)	+ [patient No. (%)]		– (patient No.)	+ [patient No. (%)]	
Age (years)				>0.05			>0.05
≤48	6	1	5(83.3)		1	5(83.3)	
>48	34	9	25(73.5)		3	31(91.2)	
Histological type				>0.05			<0.05
Serous	25	7	18(80.0)		0	25(100)	
Mucous	15	6	9(66.7)		4	11(73.3)	
Clinical stage				<0.05			<0.05
I+II	15	7	8(53.3)		4	11(73.3)	
III+IV	25	3	22(88.0)		0	25(100)	
Pathologic grade				<0.05			
G1	6	3	3(33.3)		3	3(33.3)	<0.05
G2	10	2	8(80.0)		1	9(90.0)	
G3	24	3	21(87.5)		0	24(100)	
Tumor metastasis				<0.05			>0.05
Yes	13	0	13(100)		2	11(84.6)	
No	27	10	17(63.0)		2	25(92.6)	

is closely correlated with tumor cell growth, proliferation, invasion, and metastasis.[2]

Many overseas studies have reported the relationship between OPN and ovarian cancer. It was believed that OPN highly expresses in ovarian epithelial carcinoma. Rosen *et al*.[3] found that there was no expression of OPN in normal ovarian epithelial cells, while the expression of OPN increased in ovarian cystadenoma, borderline ovarian neoplasm, and serous ovarian cancer, successively. Using immunohistochemistry, Kim *et al*.[4] detected the expression of OPN in invasive and borderline ovarian neoplasms. The results showed that the intensity of OPN expression in both invasive and borderline ovarian neoplasms was significantly higher than in benign ovarian neoplasms or normal ovarian tissue. Our results also showed that the intensity of OPN expression in malignant ovarian neoplasms was significantly higher than either in borderline or benign ovarian neoplasms, and the intensity of OPN expression in the latter two was higher than in normal ovarian tissue without significant differences. Our results were in concordance with other published results. All results suggest that OPN plays an important role in tumorigenesis and the development of ovarian epithelial neoplasms.

Coppola *et al*.[5] reported that OPN overexpressed in a variety of malignant tumors (stomach cancer, colon cancer, kidney cancer, pancreatic cancer, lung cancer, endometrial cancer, cervical cancer, ovarian cancer, and head and neck squamous cell carcinoma). Denhardt *et al*.[6] reported that OPN produced by both tumor cells and stromal cells could enhance the metastatic capacity of tumor cells. The experimental results in the present study suggest that the expression of OPN in stage III-IV cancer tissue was significantly higher than in stage I-II cancer tissue. The expression of OPN in poorly differentiated cancer tissue was significantly higher than in highly differentiated cancer tissue. In addition, OPN expression in carcinomas with lymph node metastasis was significantly higher than that in carcinomas without lymph node metastasis ($P < 0.05$). Ito *et al*.[7] also confirmed that the expression of OPN was significantly correlated with lymph node metastasis, the invasion of lymphoid organs, and pathologic stage, and associated with poor prognosis of patients with OPN expression.

Enhanced expression of OPN may involve in the progress and metastasis of malignant tumors by promoting tumor-cell growth, inducing local angiogenesis, inhibiting the inducing effect of microenvironments on apoptosis, enhancing metastasis in lymph nodes, and so on. Recently, Hurst *et al*.[8] showed that MDA-MB-435 cells secreted high levels of OPN, while the expression of OPN in the MDA-MB-435 cells transfected with breast cancer metastasis suppressor factor-1 reduced significantly and metastasis was inhibited. That further suggests that OPN plays a vital role in the metastasis of malignant tumors. But so far, the role of OPN in tumorigenesis, the development and metastasis of ovarian cancer, and its exact mechanism remains unclear and further studies will be necessary.

B7-H4 and tumors

T-cell mediated immunity played an important role in antitumor immune responses. The activation of T cells requires not only T-cell receptor (TCR)-mediated antigen signaling but also a second signal provided by cosignaling molecules. B7 family members, important cosignaling molecules, not only provide costimulatory signals that the activation of T-cells require but also the cosuppression signals that constrain and weaken the activation of T cells.

B7-H4, one of the recently discovered B7 family members, can negatively regulate T-cell immune response by inhibiting T-cell proliferation, the production of cytokines, and cell-cycle progression. High expression in a variety of tumor tissues calls attention to its role in tumorigenesis and the development of cancer. B7-H4 is considered to be a new tumor marker that is correlated with the immunologic escape of tumor cells. Therefore, B7 family members can be used for cancer immunotherapy. B7-H4 is a negative-regulation gene of T-cell immune response discovered by Sica *et al*.[9] using bioinformatics methods in 2003. B7-H4 is localized on chromosome 1, encodes a 282-amino-acid-long protein and can inhibit the cellular immune responses when combined with the T-cell receptor.

The B7-CD28 pathway is one of early signals of T-cell activation. The effects of T-cell mediated immunosuppression followed by the binding of the B7-H4 protein and its receptor cannot be reversed, even through CD28 costimulation.[9-10] Therefore, B7-H4 plays an important role in T-cell mediated immunosuppression responses. Although not discovered for a long time, B7-H4 has been identified as correlated with tumorigenesis and the development of various types of cancer. At present, it is believed that the expression of B7-H4 in tumors is an important mechanism for tumor cells escaping antitumor immunity.

After detecting concentrations of B7-H4 in tissue lysates from ovarian cancer with the enzyme-linked immunosorbent assay (ELISA), Simon *et al*.[11] found that an increase in the malignancy was accompanied by an increased level of B7-H4. In our study, 95 ovarian samples were analyzed using immunohistochemical methods. The results also found that the expression of the B7-H4 protein in malignant ovarian epithelial neoplasms was significantly higher than in benign and borderline ovarian epithelial neoplasms and its expression was correlated with the malignant types of ovarian cancer. The expression rate in serous carcinoma was 100%, while the expression rate was relatively low in mucinous carcinoma. The differences in B7-H4 expression between mucinous and nonmucinous ovarian cancer were also found in the detection of other markers of ovarian cancer,[12] which may relate to significant differences in signal pathways between mucinous and nonmucinous carcinoma, suggesting that there may be important differences in the mechanisms of tumorigenesis between mucinous and nonmucinous ovarian cancer.

Through the analysis of B7-H4 protein expression in 40 cases of epithelial ovarian cancer combined with clinical and pathologic data in this study, we found that its expression was correlated with clinical stage and pathologic grade, and there was higher expression in moderate- to advanced-stage than in early-stage

ovarian cancer and higher expression in poorly differentiated tissue than in well-differentiated tissue. In addition, the expression of the B7-H4 protein in ovarian cancer was independent of clinicopathologic factors such as age and lymph node metastasis. Salceda *et al.*[13] found that overexpression of B7-H4 in human ovarian cancer cell lines with low expression of B7-H4 could promote tumor growth in severe combined immunodeficiency (SCID) mice and inhibit tumor-cell apoptosis, suggesting that increased expression of the B7-H4 protein may lead to tumor antigenic escape that may be involved in ovarian tumorigenesis, further suggesting that B7-H4 may be one of promoter genes for ovarian cancer.

In addition, because there are several markers simultaneously in the same tumor and the same markers in various tumors, the efficiency of single-target detection is unsatisfactory. In this study, we obtained satisfactory results with combined detection of OPN and B7-H4 expression levels in ovarian tissue. Individual sensitivities of OPN and B7-H4 in the epithelial ovarian cancer group were 75% and 90%, respectively; the sensitivity of combination detection was 95%, suggesting that combined detection can improve diagnostic sensitivity of epithelial ovarian cancer.

In this study, through detecting the expression of OPN and the B7-H4 protein in epithelial ovarian neoplastic tissue, we found that the expression levels of OPN and B7-H4 significantly increased with tumorigenesis and cancer development, suggesting that both play important roles in tumorigenesis and the development of malignant ovarian neoplasms. The sensitivity increased significantly with the combined detection of the expression in ovarian cancer. Therefore, OPN and B7-H4 can be used as clinical diagnostic markers with some clinical significance in the early detection and early diagnosis of ovarian cancer.

References

[1] Senger DR, Wirth DF, Hynes RO. Transformed mammalian cells secrete specific proteins and phosphoproteins [J]. Cell, 1979,16(4):885–893.

[2] Bao LH, Sakaguchi H, Fujimoto J, et al. Osteopontin in metastatic lesions as a prognostic marker in ovarian cancers [J]. J Biomed Sci, 2007,14(3): 373–381.

[3] Rosen DG, Wang L, Atkinson JN, et al. Potential markers that complement expression of CA125 in epithelial ovarian cancer [J]. Gynecol Oncol, 2005,99(2) :267–277.

[4] Kim JH, Skates SJ, Uede T, et al. Osteopontin as a potential diagnostic biomarker for ovarian cancer [J]. JAMA, 2002,287(13):1671–1679.

[5] Coppola D, Szabo M, Boulware D, et al. Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histologies [J]. Clin Cancer Res, 2004,10(1):184–190.

[6] Denhardt DT, Mistretta D, Chambers AF, et al. Transcriptional regulation of osteopontin and the metastatic phenotype: evidence for a ras - activated enhancer in the human open promoter [J]. Clin Exp Metastasis, 2003,20 (1):77–84.

[7] Ito T, Hashimoto Y, Tanaka E, et al. An inducible short-hairpin RNA vector against osteopontin reduces metastatic potential of human esophageal squamous cell carcinoma in vitro and in vivo [J]. Clin Cancer Res, 2006,12 (4):1308–1316.

[8] Hurst DR, Xie Y, Vaidya KS, et al. Alterations of BRMS1-ARID4A interaction modify gene expression but still suppress metastasis in human breast cancer cells [J]. Biol Chem, 2008,283 (12):7438–7444..

[9] Sica GL, Choi IH, Zhu G, et al. B7–H4, a molecule of the B7 family, negatively regulates T cell immunity [J]. Immunity, 2003,18(6):849–861.

[10] Choi ZH, Zhu GL, Sica GL, et al. Genomic organization and expression analysis of B7–H4, an immune inhibitory molecule of the B7 family [J]. Immunol, 2003,171(9):4650–4654.

[11] Simon I, Zhuo S, Corral L, et al. B7–H4 is a novel membrane –bound protein and a candidate serum and tissue biomarker for ovarian cancer [J]. Cancer Res, 2006,66 (3):1570–1575.

[12] Ordonez NG. Application of mesothelin immunostaining in tumor diagnosis [J]. Am J Surg Pathol, 2003,27(1):1418–1428.

[13] Salceda S, Tang T, Kmet M, et al. The immunomodulatory protein B7–H4 is overexpressed in breast and ovarian cancers and promotes epithelial cell transformation [J]. Exp Cell Res, 2005,306(1):128–141.