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Original Article

Expression of B7-H4 in ovarian cancer and its clinical significance

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Abstract: We designed the study to investigate B7-H4 expression in ovarian carcinoma tissues and the correlation between B7-H4 expression and clinicopathological characteristics. We detected B7-H4 expression by immunohistochemical test in the tissues of 65 ovarian carcinoma cases and 40 controls. The correlation between each independent clinicopathological factor and B7-H4 expression was analyzed. The positive rates of B7-H4 in serous cystadenocarcinoma, borderline serous cystadenoma and serous cystadenoma were 87.8%, 23.0% and 0%, respectively. There was statistical difference among the positive rates. B7-H4 positive staining was significantly correlated with pathological grade and lymph node metastasis. B7-H4 expression scores were 5.75, 7.5 and 10.0 in well differentiated, moderately differentiated and poorly differentiated tissues, respectively. The differences among three groups were statistically significant. The scores of cases with lymph node metastasis and without lymph node metastasis were 7.75 and 5.50. There was significant difference between them. B7-H4 is involved in the carcinogenesis and development of ovarian carcinoma and is possible to be a candidate biomarker for diagnosing ovarian carcinoma.

Keywords: B7-H4, ovarian carcinoma, co-stimulatory

Introduction

Ovarian cancer is the most common malignant tumor of the female reproductive system with a 5-year survival rate of as low as 20%-30%. Although combination therapy of surgical treatment, radiotherapy, and chemotherapy has been commonly used in recent years, the survival rate of patients with ovarian cancer has not been significantly improved [1]. Therefore, to study the developmental mechanisms underlying ovarian cancer, finding specific and sensitive tumor markers and novel therapy targets have become a research focus of gynecologic oncology.

Synergistic stimulation has an important regulatory role in activating T cells to kill tumor cells, which is involved in the occurrence and development of many solid tumors, including ovarian cancer [2]. Synergistic stimulatory molecules are abnormally expressed in various tumor tissues, whose regulating network effectively maintains the stability of the internal environment. The B7-H4 molecule is an important negative synergistic stimulatory molecule of the B7 family, which can negatively regulate the T cell immune response by inhibiting the proliferation of T cells, cytokine production, and cell cycle progression [3]. Many studies have revealed abnormally high expression of B7-H4 in tumor cells and tumor-associated macrophages in various tumor tissues, including ovarian, lung, kidney, stomach, prostate, esophageal, and breast cancer, and its expression level is closely associated with the clinical pathological features and prognosis of patients [3-6]. In the current study, we utilized immunohistochemistry (IHC) to detect B7-H4 expression in 105 cases with ovarian cancer tissue, compared the expression rate of B7-H4 among ovarian cancer tissues, and analyzed the correlation between the B7-H4 expression level in 65 cases of malignant tumor tissue and age, pathological type, stage, pathological grade, and lymph node metastasis of the patients, aiming provide a

novel theoretical basis for the early diagnosis and treatment of ovarian cancer.

Methods

Subjects

We recruited 65 newly diagnosed patients with ovarian cancer who were admitted to the Maternal and Child Health Hospital (Changzhou, China) from August 2010 to July 2014. All patients received surgical treatment with confirmed diagnosis, and complete clinical data was collected. These patients included 41 cases of serous adenocarcinoma, 3 cases of mucinous adenocarcinoma, 4 cases of endometrioid adenocarcinoma, 4 cases of clear cell carcinoma, 3 cases of granular cell tumor, and 10 cases of metastatic adenocarcinoma. Patients in the control group were admitted during the same period for surgical treatment, with pathological confirmed diagnoses of borderline serous cystadenoma (26 cases) or benign ovarian serous cystadenoma (14 cases). All pathological results were independently diagnosed by two doctors. The tissue samples were fixed with 10% formalin and embedded in paraffin immediately after being obtained in surgery. None of the patients received any preoperative chemotherapy or hormone therapy.

Reagents

IHC staining was performed with rabbit anti-human B7-H4 monoclonal antibody as the primary antibody (Novus Biologicals, USA), rat and rabbit universal secondary antibodies, citrate antigen retrieval solution, hematoxylin, and neutral resin used as sealant for counterstaining (Fuzhou Maixin Biotechnology).

Experimental methods

Staining was carried out using the ElivsionTM IHC staining method. After dewaxing and hydration, the paraffin slices were immersed in citrate buffer (10 mmol/L, pH 6.0), and heated in a 100°C water bath for 30 min for antigen repairing. After cooling, the slices were soaked in 3% H_2O_2 for 30 min to deactivate endogenous peroxidase. The slices were then immersed and rinsed with PBS (pH 7.4) 3 times for 5 min each. Primary antibody (CD11c rabbit anti human monoclonal antibody, 1:150 dilution) was then added, and the slices were kept at 4°C overnight. The slices were immersed and rinsed again in PBS (pH 7.4) 3 times for 5 min each, after which secondary antibody (mouse/rabbit universal secondary antibodies) was added, and the slices were kept at room temperature for 30 min. We then used PBS to wash off the secondary antibody, followed by the addition of DAB for color development, hematoxylin counterstaining, and differentiation by ethanol with 0.1% hydrochloric acid. The slices were mounted with neutral resin after gradient ethanol dehydration and drying. PBS was used instead of the primary antibody as a negative control, and breast cancer tissue was used as a positive control.

Evaluation of results

Yellowish-brown granular spots in the cytoplasm or on the cell membrane of ovarian cancer cells indicated positive staining for B7-H4 expression. Five regions were randomly selected under a 100× magnification high-power lens, and we counted the number of cells showing positive staining in the cytoplasm or on the cell membrane as follows: 0 points for tissues without positive staining, 1 point for tissues with positive staining in 1%-10% cells, 2 points for ~11%-50%, 3 points for ~51%-80%, and 4 points for ~81%-100%. The intensity of positive staining was also assessed as follows: 0 for negative staining, 1 point for weak positive staining, 2 points for moderate positive staining, and 3 points for strong positive staining. Additionally, the IHC score of the samples was calculated by multiplying the above 2 scores. The current study used 4 as the reference value for the diagnosis of ovarian cancer by B7-H4 expression. Negative expression was determined by a score ≤4, and positive expression was determined by a score >4.

Statistical analysis

All data was analyzed using SPSS v.13.0 (Chicago, IL, USA). Categorical data was analyzed using the χ^2 test. B7-H4 levels in samples with different clinical characteristics were compared with the rank sum test, with $P<0.05$ considered as statistically significant.

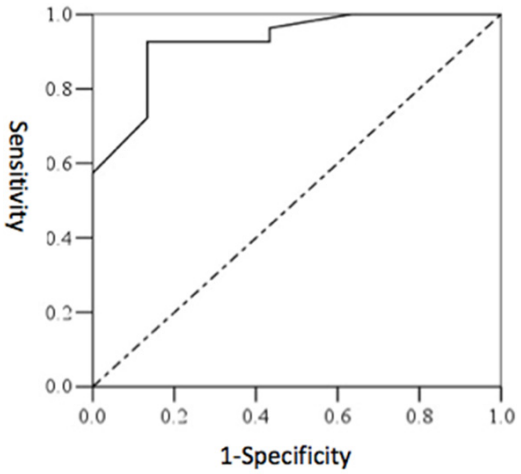

Figure 1. ROC curve for B7-H4 diagnosis of ovarian cancer.

Results

Establishment of a positive reference value of B7-H4 expression

When the B7-H4 reference value was set at 4, the sensitivity, specificity, Youden index, and area under the ROC curve for diagnosis of ovarian cancer were 92.6%, 86.7%, 0.793, and 0.927, respectively (**Figure 1**). The diagnosis efficiency was highest at this positive reference value.

Expression of B7-H4 in different groups of ovarian serous tumors

B7-H4 was mainly expressed in the cytoplasm and on the membrane of ovarian cancer cells, which was seen as diffuse brown and yellow granular staining (**Figure 2**). B7-H4 positive expression rates in ovarian serous cystadenomas, borderline serous cystadenomas, and slurry cystic gland tumor tissues were 0% (0/14), 23% (6/26), and 87.8% (36/41), respectively. The expression rates among the three groups were significantly different from each other (χ^2=36.85, P<0.05, **Table 1**).

B7-H4 expression in ovarian cancer tissues

Among the 54 cases of ovarian cancer tissues, the positive B7-H4 expression rates in slurry cancer, endometrial cancer, mucus carcinoma, clear cell cancer, granular cell carcinoma, and metastatic adenocarcinoma were 87.8% (36/41), 100% (3/3), 100% (4/4), 100% (4/4), 100% (3/3), and 100% (10/10), respectively. No statistical analysis was carried out due to the limited case number of each kind of tumor.

The relationship between the expression of B7-H4 and clinical pathological parameters in ovarian cancer tissues

Among the 54 cases of ovarian cancer, B7-H4 expression levels were correlated with the pathological grading and lymph node metastasis of the ovarian cancer tissues, but not with the pathological type or FIGO stage of ovarian cancer, or with the age of the patients. The median expression scores of B7-H4 in the high, moderate and low differentiated tumor tissue groups were 5.75 (3.0-9.0), 7.5 (3.5-12.0) and 10 (3.5-12.0), respectively, and the B7-H4 expression level increased with a decrease in tumor cell differentiation. B7-H4 expression in the low differentiation group was significantly higher than that in the moderate differentiation group, while the B7-H4 expression level in the moderate differentiation group was significantly higher than that of the high differentiation group, with statistically significant differences among the three groups (χ^2=9.61, P<0.05, **Table 2**). The median B7-H4 expression score in the ovarian cancer tissues with lymph node metastasis was 7.75 (3.0-12.0), which was significantly higher than that (5.50) of cases without lymph node metastasis (4.5-7.5, **Table 2**; Z=2.23, P<0.05).

Discussion

The immune system participates in the occurrence and development of tumors, and tumor-infiltrating immune cells including T cells, B cells, macrophages, dendritic cells, and mast cells make up the immune system within the microenvironment of tumor [7]. During an immune response, synergistic stimulatory molecules play an important role in its effective stimulation, moderating effects, and timely termination. The absence of synergistic stimulatory molecules would lead to the regulatory unresponsiveness of T cells and specific immune tolerance and may even induce cell apoptosis. Optimal synergistic stimulatory signals can reduce the demand of first signal during T cell activation, while inhibitory synergistic stimulatory signals can weaken the immune response or lead to immune tolerance.

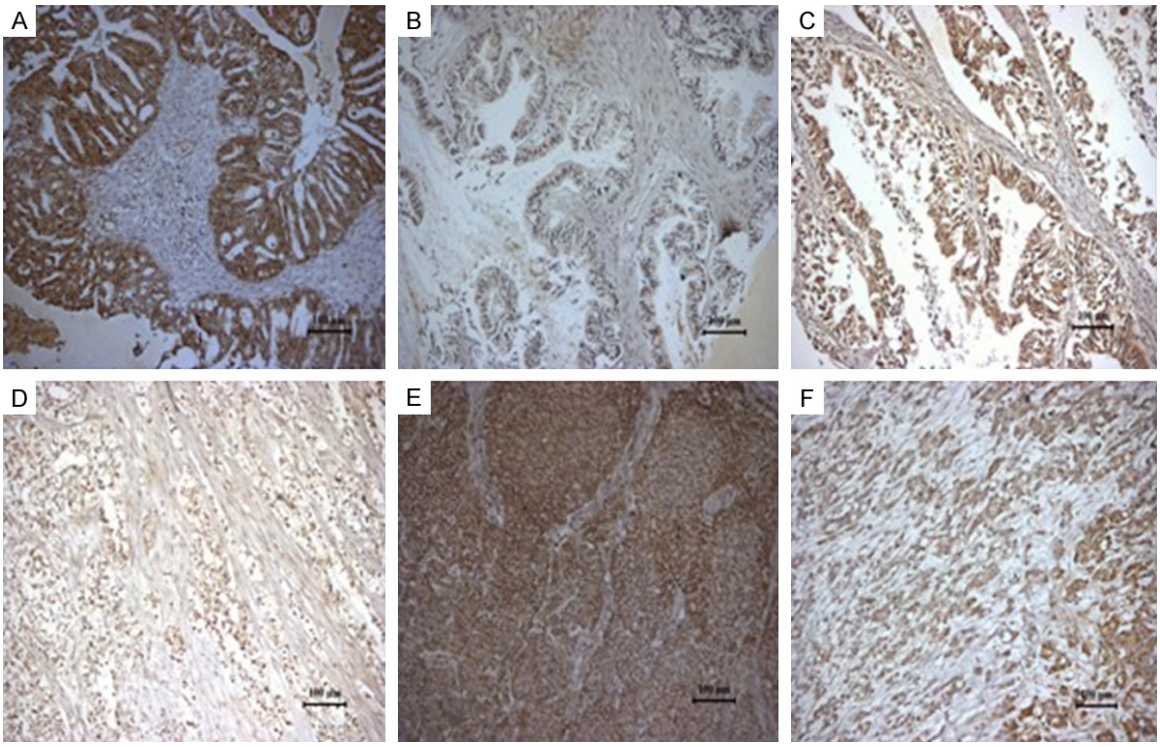

Figure 2. Expression of B7-H4 was mainly localized in the cytoplasm and cell membrane of tumor cells, showing diffusive brown granular staining. A. Serous adenocarcinoma with positive expression of B7-H4; B. Mucinous adenocarcinoma with positive expression of B7-H4; C. Endometrioid adenocarcinoma with positive expression of B7-H4; D. Transparent cell carcinoma with positive expression of B7-H4; E. Granulosa cell tumor with positive expression of B7-H4; F. Ovarian metastasis cancer with positive expression of B7-H4. All magnifications are 100×.

Table 1. Comparison of the positive expression rate of B7-H4 in ovarian serous tumors

Group	N	B7-H4 positive cases (%)	χ^2	*P*
Ovarian serous cystadenoma (malignant group)	41	36 (87.8)	36.85	0.000*
Ovarian borderline serous cystadenoma (borderline group)	26	6 (23.0)		
Ovarian slurry cystic gland tumor (benign group)	14	0		

*: using corrected χ^2 test.

B7-H4 is an important member of the B7 negative regulation family, which plays an important role in the process of tumor immune escape.

The positive B7-H4 expression rate among the tissue specimens from 65 cases with ovarian cancer in the current study was higher than benign tumor tissues, suggesting that B7-H4 is involved in the occurrence ovarian cancer and may become a new tumor marker of ovarian cancer with diagnostic value, providing a target for gene therapy of ovarian cancer, consistent with the results of another recent study [8].

We found that the expression rate of B7-H4 was high in malignant tumor tissue, low in borderline tumor tissue, and negative in benign tumor tissues. Cheng et al. [9] established the B7-H4-negative SKOV3 ovarian cancer cell line and constructed a tumor-bearing mouse model. After transfection with B7-H4, the tumor grew rapidly. Salceda et al. [10] confirmed that B7-H4 was up-regulated in both breast and ovarian cancer cell lines using IHC. In addition, *in vitro* studies showed that knockdown of B7-H4 using small interfering RNA induced tumor cell apoptosis. In contrast, increased expression of B7-H4 in cell lines significantly inhibited cell apoptosis. These results suggest that B7-H4 may play an important role in the occurrence of ovarian cancer, and it could become a candi-

Table 2. Relationship between B7-H4 expression levels and different clinical pathological parameters in malignant ovarian cancer cells

Clinical pathological parameters		Total case number	B7-H4 (Min-Max)	Z	P
Age	≤50	43	7.50 (3.0-12.0)	0.48	0.630
	>50	22	7.50 (3.0-12.0)		
Pathological types	Serous adenocarcinoma	41	8.25 (3.0-12.0)	1.67	0.232
	Others	24	6.75 (4.5-12.0)		
Lymph node metastasis	Yes	10	7.75 (3.0-12.0)	2.23	0.022
	No	55	5.50 (4.5-7.5)		
Staging	Stage I-II	52	7.50 (3.0-12.0)	0.85	0.366
	Stage III-IV	13	9.0 (5.5-10.0)		
Differentiation	High	12	5.75 (3.0-9.0)	9.61*	0.009
	Medium	17	7.5 (3.5-12.0)		
	Low	36	10.0 (3.5-12.0)		

*: Kruskal-Wallis Test.

date marker for the differential diagnosis of benign and ovarian cancer.

Positive expression of B7-H4 was observed in 36 out of 41 samples with early-stage ovarian serous carcinoma, indicating that B7-H4 is expressed in ovarian cancer beginning at an early stage. Two studies by Simon et al. [11, 12] showed that the expression level of B7-H4 in ovarian cancer tissue was consistent with the expression level of B7-H4 in the serum of patients, and the expression of B7-H4 in tissues could reflect the preoperative serum level of the patients. These findings suggest that the detection of serum B7-H4 level may be helpful for the early detection of ovarian cancer.

Saleeda et al. [10] confirmed that the expression of B7-H4 on tumor cell surfaces has a direct promoting effect on the malignant transformation of epithelial cells in animal experiments. By exploring the relationship between B7-H4 expression level in ovarian cancer tissues and the clinical pathology characteristics of patients, our study found that the B7-H4 expression intensity was different in ovarian cancer tissues with different degrees of differentiation, and B7-H4 became over-expressed with decreasing histological differentiation, indicating that B7-H4 may promote the occurrence and development of ovarian cancer. Therefore, B7-H4 expression levels can reflect the differentiation degree of tumor tissues and indicate poor prognosis. The median B7-H4 expression level in ovarian cancer tissues in cases with lymph node metastasis was significantly higher than that in cases without lymph node metastasis, suggesting that the expression of B7-H4 promotes tumor cell metastasis through a lymphatic pathway. Our study found that there was not a significant correlation between B7-H4 expression levels in ovarian cancer tissues and patient age, suggesting that B7-H4 expression is not affected by the menopausal status of the patients. We also found that expression of B7-H4 in ovarian cancer tissues was not correlated with FIGO stage or histological type of the patients.

In summary, the expression of B7-H4 is related to the occurrence and development of ovarian cancer and is expected to become a new molecular marker for the evaluation of the biological behavior of these tumors. However, the effects of B7-H4 in the development of ovarian cancer and its value in clinical practice need to be further verified in studies with larger sample sizes.

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Disclosure of conflict of interest

None.

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