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The expression of B7-H3 and B7-H4 in human gallbladder carcinoma and their clinical implications

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Abstract. – OBJECTIVE: To investigate the expression of B7-H3 and B7-H4 and their clinical implications in human gallbladder carcinoma.

PATIENTS AND METHODS: The expression of B7-H3 and B7-H4 in the 252 samples (126 cases of chronic cholecystitis and 126 cases of gallbladder cancer) was detected by the streptavidin-peroxidase immunohistochemical method, and their associations with tumor classification, clinical grade, and recurrence were assessed.

RESULTS: In chronic cholecystitis tissue, B7-H3 and B7-H4 were not detected. In 126 cases of gallbladder carcinoma, the positive rates of B7-H3 and B7-H4 expression were 66.67% and 69.05% respectively ($p < 0.05$). The positive rate of B7-H3 in the primary-onset group was 53.57%, and that in recurrence group was 92.86% ($p < 0.05$). The positive rate of B7-H4 in the primary-onset group was 85.19%, and that in recurrence group was 40.00% ($p < 0.05$). Expression of B7-H3 was consistent with B7-H4 expression in gallbladder carcinoma.

CONCLUSIONS: B7-H3 and B7-H4 were upregulated in gallbladder cancer; the high expression of B7-H3 may contribute to the early diagnosis of gallbladder carcinoma and the assessment of postoperative survival and recurrence. B7-H4 may play an important role in the incidence of gallbladder cancer. B7-H3 and B7-H4 may play a synergetic role in gallbladder carcinoma. Combined tests were available for the diagnosis, degree assessment and prognosis of gallbladder carcinoma, which may be a new target for molecular targeted therapy of gallbladder carcinoma.

Key Words:
Gallbladder carcinoma, B7-H3, B7-H4, Immunohistochemistry.

Introduction

Patients with gallbladder cancer are often in advanced stage when admitted, with low resection rate, poor prognosis and very low survival rate of five years. With advances in polyclinics technology, the overall survival rate of gallbladder cancer has increased significantly, but compared to other malignancies, the prognosis remains poor, due to the high rates of recurrence and metastasis even after radical resection of gallbladder cancer[1]; and gallbladder tumor cells are not sensitive to traditional radiochemotherapy. So, how to delay or block the gallbladder cancer progress has attracted more and more attention. B7 family, kind of co-stimulatory factors, is the focus of current research of tumor markers, including B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, and B7-H4, of which B7-H3 and B7-H4 are research hotspots in recent years.

Domestic and foreign scholars conducted more and more research on the roles of B7-H3 and B7-H4 in the incidence, invasion and metastasis of many malignant tumors. It has been found that B7-H3 and B7-H4 had high positive rates in non-small cell lung cancer[2-4], gastric cancer[5,6] and breast cancer[7,8], and the expression levels were closely correlated with tumor recurrence, metastasis and overall survival rates. But there are no related reports in gallbladder cancer. In this study, the expression of B7-H3 and B7-H4 in gallbladder cancer was detected by immunohistochemical method, and their associations with age, gender, histological grade, clinical stage, the initial issuance or recurrence, tumor

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size, lymph node metastasis, liver invasion, survival time were assessed, in order to explore their implications in tumor incidence, development, prognosis and treatment.

Patients and Methods

Patients

Respectively 126 cases of gallbladder cancer and 126 cases of chronic cholecystitis were collected in our hospital from October 2004 to October 2014, each including 48 males and 78 females; gallbladder cancer was taken as the study group, aged between 32 and 77 years, with a median age of 59.8 years old; chronic cholecystitis was taken as the control group, aged between 31 and 75 years, with a median age of 58.3 years old. Follow-up and survival time were recorded from postoperative to October 2014. The study protocol was approved by the Ethics Review Board of our hospital. We have obtained written informed consent from all study participants. All of the procedures were done in accordance with the Declaration of Helsinki and relevant policies in China.

Inclusion criteria: (1), patients diagnosed and confirmed according to the WHO (2000) gallbladder cancer TNM staging system; (2) patients receiving the treatment of cholecystectomy; (3), patients without preoperative anti-tumor therapy; (4), patients with comprehensive clinical data and follow-up data.

Exclusion criteria: (1), follow-up was unsuccessful or exact survival time cannot be obtained; (2), survival but halfway lost; and (3), deaths caused by non-gallbladder cancer; (4), at the end of follow-up, patients were still alive.

Observation indicators: age, gender, histological grade, clinical stage, the initial issuance or recurrence, tumor size, lymph node metastasis, liver infiltration and postoperative survival time.

The Main Equipments and Reagents

OLYMPUS® CK40 inverted fluorescence microscope (Olympus, Shinjuku District, Tokyo, Japan); MKJ-J1-8 experimental microwave (Shandong Qingdao Michael Granville Co. Ltd. Qing-dao, Shandong Province, China); CUT5062 semiautomatic paraffin sector (SLEE company, Mainz, Germany); photographic collection system (Leica, Oskar, Barnack, Germany); a digital camera (Canon, Roppongi, Tokyo, Japan); (1) 3-aminopropyl triethoxysilane (KH-550); Human B7-H3 Allophycocyanin MAb (Clone 185504) and Mouse IgG1 (R & D Company, Long Beach, CA, USA); Mouse B7-H4 MAb (Clone 297219) and Rat IgG2B (R & D Company, Long Beach, CA, USA); UltraSensitive S-P kit and concentrated DAB kit (Wuhan Boster, Hubei Province, China).

Methods

Detection of B7-H3 and B7-H4

S-P immunohistochemical method was used to detect the expression of B7-H3 and B7-H4. Specimens of gallbladder carcinoma and chronic cholecystitis were fixed in 10% formalin solution, paraffin-embedded and sliced to 4 m sections, which were dehydrated by graded ethanol and transparent by xylene; strictly in accordance with the kit instructions, HE staining and immunohistochemical staining were performed, with mouse B7-H3 and B7-H4 monoclonal antibodies (1:100) as the primary antibodies; with Mouse IgG1 and Rat IgG2B as the secondary antibodies.

Assessment

The Criteria of Tumor Differentiation Degree

The results were assessed by two or more pathologists; referral when disagreement existed. The Edmondson I-II grade was classified as well-differentiated and III-IV grade was classified as poorly differentiated.

The positive criteria

Cells with Yellow-brown granules in the cell membrane and/or cytoplasm, good location and deeper staining than the background were judged as positive cells (Figure 1).

B7-H3 determination: five high power fields (X200) of each slice were randomly selected, judging according to the percentage of positive cells. Positive cells % = 0% (-), 0% ≤ positive cells % < 10% (+), 10% ≤ positive cells % < 50% (++), 50% ≤ positive cells % < 80% (+++), positive cells % ≥ 80% (++++); ≥ 10% was positive and < 10% was negative; Negative or weakly positive were enrolled in low expression group, moderate or strong positive were enrolled in high expression group.

B7-H4 determination: five high power fields (X400) of each slice were randomly selected, judging according to the percentage of positive

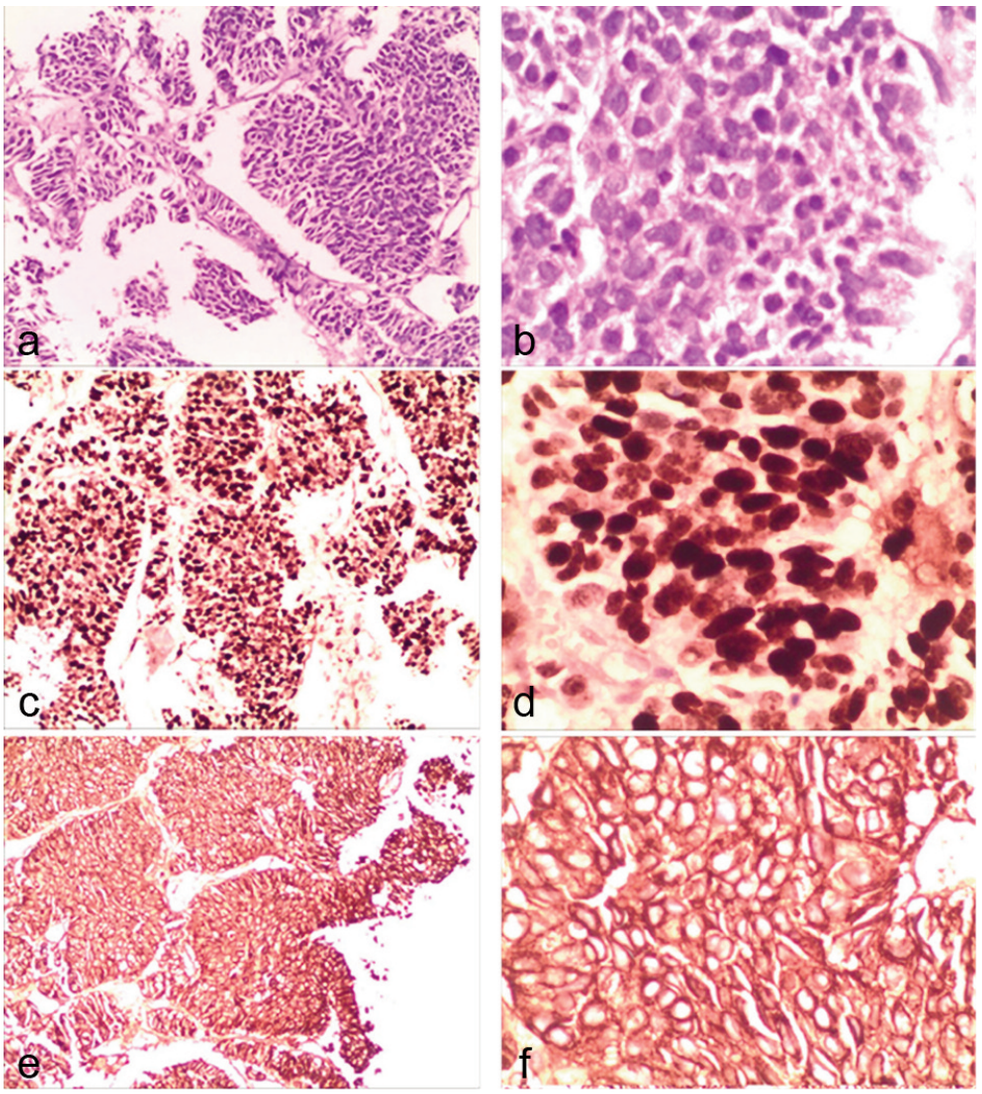

Figure 1. ***A***, HE staining of gallbladder carcinoma sample (X 200). ***B***, HE staining of gallbladder carcinoma sample (X 400). ***C***, B7-H3 positive expression in gallbladder carcinoma (X 200). ***D***, B7-H3 positive expression in gallbladder carcinoma (X 400). ***E,*** B7-H4 positive expression in gallbladder carcinoma (X 200). ***F***, B7-H3 positive expression in gallbladder carcinoma (X 400).

cells. 0% ≤ positive cells % <1 0% (-), 10% ≤ positive cells% < 40% (+), 40% ≤ positive cells% < 80% (++), positive cells% ≥ 80% (+++); ≥ 10% was positive and < 10% was negative; negative or weakly positive were enrolled in low expression group, moderate or strong positive were enrolled in high expression group.

Statistical Analysis

SPSS17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis; count data were analyzed using x^2 test, continuity correction x^2 test or Fisher's exact test; correlation analysis of two indicators was conducted using Mcnemar test; survival differences were compared by Kaplan-Mier and Log-Rank Test; all results took $\alpha = 0.05$ (bilateral) as test standard; $p < 0.05$ was considered statistically significant.

Results

General Information of Patients

The tumor diameter of 126 gallbladder cancer patients was between 0.5 and 11 cm, with an average diameter of 3.2 cm, including 69 cases

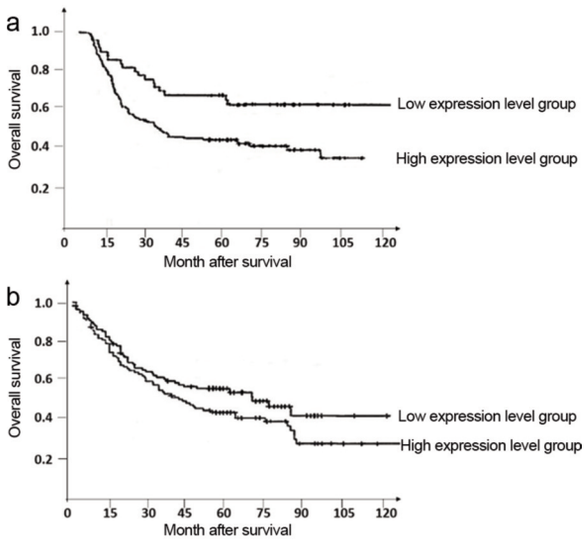

Figure 2. ***A***, Comparison of overall survival rate between B7-H3 high-expression group and B7-H3 low-expression group (Kaplan-Meier, log-rank test). ***B***, Comparison of overall survival rate between B7-H4 high-expression group and B7-H4 low-expression group (Kaplan-Meier, log-rank test).

with tumor diameter ≥ 3 cm and 57 cases with tumor diameter < 3 cm.

According to WHO gallbladder cancer TNM stage (2000), there were 27 cases at stage I, 9 cases at stage II, 63 cases at stage III and 27 cases at stage IV; Lymph node metastasis was found in 48 cases; local liver invasion was found in 54 cases.

Histopathological classification: 90 cases of NOS adenocarcinoma, 24 cases of papillary adenocarcinoma, respectively 6 cases of mucinous adenocarcinoma and gland cancer; 21 cases of high-differentiated, 72 cases of moderate-differentiated and 33 cases of poorly differentiated.

1-year survival rate of 42 patients with gallbladder cancer was 41.86%, 25.58% for 2-year survival rate, with a median survival time of 6.5 months.

Expression of B7-H3 and B7-H4 in the Specimens of Gallbladder Carcinoma and Chronic Cholecystitis

B7-H3 was found in 66.7% (84 cases) of 126 gallbladder carcinoma specimens, and there was no B7-H3 expression in 126 cases of chronic cholecystitis samples; B7-H3 expression in gallbladder carcinoma was significantly higher than that of chronic cholecystitis ($p < 0.05$) (Table I).

The positive rate of B7-H4 was 69.0% in 126 cases of gallbladder carcinoma samples, and there was no expression in 126 cases of chronic cholecystitis samples; B7-H4 expression in gallbladder carcinoma was significantly higher than that of chronic cholecystitis ($p < 0.05$) (Table II).

Relationship Between the expression of B7-H3 and B7-H4 and gallbladder Carcinoma Pathological Grades

The positive rate of B7-H3 in different pathological grades increased with the increased degree of malignancy ($p < 0.05$) (Table III).

Table I. B7-H3 expression in gallbladder carcinoma and chronic cholecystitis.

	B7-H3			
No	+	-	*p*-value	
Gallbladder	126	84	42	
Chronic cholecystitis	126	0	126	< 0.01

Note: $X^2 = 42.000$, V = 1, $p < 0.05$ was considered as statistically significant difference.

Table II. B7-H4 expression in gallbladder carcinoma and chronic cholecystitis.

	B7-H4			
	No	+	-	*p*-value
Gallbladder	126	87	39	
Chronic cholecystitis	126	0	126	< 0.01

Note: $X^2 = 44.291$, V = 1, $p < 0.05$, was considered as statistically significant differences.

Table III. B7-H3 expression in different pathological grades.

	B7-H3			
	No	+	-	*p*-value
Poor differentiation	33	30	3	
High-moderate differentiation	93	54	39	= 0.0047

Note: $X^2 = 3.941$, V = 1, $p < 0.05$ was considered as statistically significant difference.

Table IV. B7-H4 expression in different pathological grades.

	B7-H4			
	No	+	-	*p*-value
Low level of differentiation	33	12	21	
High-middle level of differentiation	93	75	18	= 0.0019

Note: $X^2 = 5.521$, V = 1, $p < 0.05$ was considered as statistically significant difference.

The positive rate of B7-H4 in different pathological grades decreased with the increased degree of malignancy ($p < 0.05$) (Table IV).

Relationship of B7-H3 and B7-H4 Expression with Clinical Stages of Gallbladder Carcinoma

B7-H3 expression rate had a positive correlation with clinical stages of gallbladder carcinoma; the positive rate increased with increasing clinical stages ($p < 0.05$) (Table V).

B7-H4 expression rate had a negative correlation with clinical stages of gallbladder carcinoma; the positive rate decreased with increasing clinical stages ($p < 0.05$) (Table VI).

Relationship of B7-H3 and B7-H4 Expression with Primary and Recurrent Gallbladder Carcinoma

The positive rate of B7-H3 in recurrence group was significantly higher than that in primary group ($p < 0.05$) (Table VII).

The positive rate of B7-H4 in recurrence group was significantly lower than that in primary group ($p < 0.05$) (Table VIII).

Table V. B7-H3 expression in different clinical stages.

	B7-H3			
	No	+	-	*p*-value
T1	27	9	18	
T2-T4	99	60	39	= 0.0041

Note: $X^2 = 5.727$, V = 1, $p < 0.05$ was considered as statistically significant difference.

Table VI. B7-H4 expression in different clinical stages.

	B7-H4			
	No	+	-	*p*-value
T1	27	27	0	
T2-T4	99	75	24	= 0.0038

Note: $X^2 = 5.135$, V = 1, $p < 0.05$ was considered as statistically significant difference.

Table VII. B7-H3 expression in primary and recurrent group.

B7-H3				
Recurrent group	No	+	-	*p*-value
	45	42	3	
Primary group	81	45	36	= 0.015

Note: $X^2 = 6.482$, V = 1, $p < 0.05$ was considered as statistically significant difference.

Table VIII. B7-H4 expression in primary and recurrent group.

B7-H4				
Recurrent group	No	+	-	
	45	18	27	*p*-value
Primary group	81	69	12	= 0.007

Note: $X^2 = 7.219$, V = 1, $p < 0.05$ was considered as statistically significant difference.

The relationships of B7-H4 and B7-H3 Expressions in Gallbladder Cancer with Other Clinicopathological Parameters

There were no statistically significant differences in B7-H3 and B7-H4 expressions between patients over 60 years old and patients under the age of 60 years old, males and females (all $p > 0.05$). There were statistically significant differences in B7-H3 expression between patients with and without lymph node metastasis, patients with and without liver invasion, patients with tumor size ≥ 3 cm and patients with tumor size < 3 cm (all $p < 0.05$), while with no statistically significant differences in BH-H4 expression (all $p > 0.05$) ($p < 0.05$) (Tables IX and X).

Relationship of B7-H3 and B7-H4 Expressions with Overall Survival Rates of Gallbladder carcinoma (Figure 2)

Overall survival rate in B7-H3 high-expression group was significantly lower.

There was no significant difference in overall survival rate between B7-H4 high-expression group and B7-H4 low-expression group.

Correlation Between B7-H3 and B7-H4 Expression in Gallbladder Carcinoma

B7-H3 expression was consistent with B7-H4 expression in gallbladder carcinoma ($p < 0.05$) (Table XI).

Discussion

This is the first application of immunohistochemistry to detect the expression level of B7-H3 and B7-H4 in the gallbladder and chronic cholecystitis cells, confirming that B7-H3 and B7-H4 were highly expressed in gallbladder. In addition, the homology of amino acid sequence in receptor domain of B7-H4 and B7-H3 ligand was 24%, indicating that both of the two may have some

Table XI. The correlation between B7-H3 expression and biological behaviors of gallbladder cancer.

B7-H4				
Recurrent group	No	+	-	
	45	18	27	*p*-value
Primary group	81	69	12	= 0.007

Note: $X^2 = 7.219$, V = 1, $p < 0.05$ was considered as statistically significant difference.

Table IX. The correlation between B7-H3 expression and biological behaviors of gallbladder cancer.

Characters	Group	No	B7H3				X^2	*p*-value
			0	+	++	+++		
Age	≥ 60	66	12	6	33	15	0.431	
	< 60	60	15	9	27	9		0.051
Sex	Male	48	12	6	18	12	0.071	
	Female	78	15	9	30	24		0.791
Lymphnode	With	48	9	3	18	24	5.048	
metastasis	Without	78	21	15	15	27		0.025
Liver	With	54	3	9	12	30	3.938	
infiltration	Without	72	15	18	18	21		0.047
Tumor	< 3 cm	57	18	12	12	15	5.815	
diameter	≥ 3 cm	69	3	9	27	30		0.016

Note: $X^2 = 7.219$, V = 1, $p < 0.05$ was considered as statistically significant difference.

Table X. The correlation between B7-H4 expression and biological behaviors of gallbladder cancer.

Characters	Group	No	B7H4				X^2	*p*-value
			0	+	++	+++		
Age	≥ 60	66	12	6	30	18	0.293	
	< 60	60	15	6	27	12		0.588
Sex	Male	48	9	6	18	15	0.001	
	Female	78	15	9	30	24		1.000
Lymphnode	With	48	6	9	15	18	0.098	
metastasis	Without	78	15	12	27	24		0.754
Liver	With	54	6	3	21	24	3.008	
infiltration	Without	72	18	12	24	18		0.083
Tumor	< 3 cm	57	15	9	21	12	2.019	
diameter	≥ 3 cm	69	6	9	27	27		0.155

Note: X^2 test, V = 1, $p < 0.05$ was considered statistically significant different.

Table XI. The correlation between B7-H3 and B7-H4 expression in gallbladder carcinoma.

B7H3	B7H4		*p*-value
	+	-	
+	66	18	
	30	12	0.032

Note: $p < 0.05$ was considered significantly different (Chi square test of matched fourfold tables: Mcnemar test).

consistency in biological function. Therefore, in this study we did some research on therelevance of B7-H3 and B7-H4 in order to define whether the two interact with each other in the development process of carcinogenesis. The results showed that the negative expression level of B7-H3 and B7-H4 in gallbladder tissue had consistency, with significant correlation and statistically significant difference ($p < 0.05$), indicating that the two may have a synergistic effect in the occurrence and progression of gallbladder. The combination of B7-H3 and B7-H4 had benefit for early diagnosis and postoperative prognosis of gallbladder cancer.

The overall survival rate was lower in high expression level of B7-H3 gallbladder group than that in low expression group, indicating that immune evasion may play an important role in the development process of the gallbladder. There was no significant difference between high expression of B7-H4 gallbladder group and low expression level group in the overall survival rate, indicating that abnormal expression of B7-H4 in gallbladder may make normal macrophages have a suppression function, so that it may play a role in the progress that tumor cells evade the attack

of immune system. The mechanism of B7-H3 and B7-H4 was unclear, so further studies need to be taken.

The pathways and receptors of B7-H3 and B7-H4 were still unclear. Therefore, further studies of pathways and receptors should be focus on in future. In addition, we need to expand the numbers and clinical characteristics of the patients, such as increasing research on the expression level of B7-H3 and B7-H4 in gallbladder polyps and adjacent normal tissues, and studies including the indicator of jaundice and the presence of gallstones.

Conclusions

We used immunohistochemistry to detect B7-H3 and B7-H4 expression in 126 cases of gallbladder cancer patients, in order to observe their relationships with various clinicopathological parameters of gallbladder carcinoma and the correlation between the two; now the results were concluded as follows:

1. The up-regulated B7-H3 in gallbladder cancer was closely related to the patient's pathological grade, clinical stage, tumor size, lymph node metastasis, liver infiltration and the overall survival, regardless of age and gender. The high expression of B7-H3 may contribute to the early diagnosis of gallbladder carcinoma and the assessment of postoperative survival and recurrence.

2. The up-regulated B7-H4 was in carcinoma of gallbladder closely related to the patient's pathological grade and clinical stage, regardless of the patient's age, gender, tumor size, lymph node metastasis, liver infiltration and overall survival. B7-H4 may play an important role in the incidence of gallbladder cancer.

3. B7-H3 and B7-H4 may play a synergetic role in gallbladder carcinoma. The two-combined test was available for the diagnosis, degree assessment and prognosis of gallbladder carcinoma, which may be a new target for molecular targeted therapy of gallbladder carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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