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Clinicopathologic and Prognostic Significance of CD24 in Gallbladder Carcinoma

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Abstract CD24, a small cell surface protein, has emerged as a novel oncogene and prognostic factor for poor outcomes in many human cancers. However, the association of CD24 expression pattern in gallbladder carcinoma with patients' survival has not been reported. To shed light on this problem, we performed an analysis on the relationship between CD24 expression and prognostic parameters in gallbladder carcinoma. CD24 expression was examined immunohistochemically on paraffin-embedded tissue specimens from 207 patients who underwent surgical treatment for gallbladder carcinoma in the period between January 2004 and May 2009. CD24 positive expression was found in 78.7% (163/207) of the tumor samples. It tended to be associated positively with tumor histological grades and pT stages. Kaplan-Meier curves showed that CD24 positive expression was significantly related to decreased overall survival ($p<0.01$). Multivariate analysis, including CD24 expression, pT stage, tumor grade, and resection margin involvement, showed that CD24 positive expression was an independent prognostic marker in gallbladder carcinoma ($p=0.02$; relative risk=1.6). Our data demonstrate for the first time that CD24 is an important marker of malignancy and poor prognosis in gallbladder carcinoma. Its detection combined with cancerous staging may increase the ability of investigators to predict the prognosis of patients with gallbladder carcinoma. Furthermore, the CD24 antigen represents an attractive target for specific therapies with monoclonal antibodies in patients with CD24-overexpressing gallbladder carcinoma, so the detection of CD24 may help clinicians select patients likely to benefit from novel molecular therapies.

Keywords Gallbladder carcinoma · CD24 · Clinicopathology · Prognosis

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Introduction

Gallbladder carcinoma is a very lethal malignant neoplasm because of its early metastasis, strong invasion and poor prognosis. Its incidence has been increasing in recent years [1]. Despite the improvement of diagnostic modalities, most cases with gallbladder carcinoma are diagnosed at advanced stages, because no typical symptoms and signs are available at early stage, which results in poor prognosis even after surgical resection [2]. Only less than 5% patients with gallbladder carcinoma may survive [3]. Therefore, it is very important to estimate the malignant degree and invasion tendency in order to guide clinical diagnosis and treatment of this disease.

CD24, a mucin-like adhesion molecule, has recently raised attention and lent substantial improvements for our understanding of tumor biology [4]. It was first introduced as a glycosylphosphatidylinositol (GPI)-linked B-cell-related antigen in humans [5]. CD24 is a small, heavily-

glycosylated protein core that consists of 27 amino acids, and it is attached to cell membranes by a GPI anchor [6, 7]. Because it is the ligand of P-selectin and the adhesion receptor found in activated endothelial cells and platelets, CD24 is considered to be associated with tumor metastasis. Moreover, it has been described as a diagnostic molecular marker of malignant tumor and for patient prognosis. Schindelmann et al [8] demonstrated that there was a highly significant dis-regulated CD24 expression in invasive tumor cell lines by using CD24 mRNA real-time RT-PCR and flow-cytometric analyses. Senner et al [9] reported a more locally aggressive behavior of CD24-positive gliomas in a mouse model. In addition, recent studies also have reported CD24 expression in non-small cell lung cancer [10], breast cancer [11], and prostatic cancer [12] was being closely related with the tumor metastasis, the survival rate of patients, and the recurrence rate of tumor.

Because gallbladder carcinoma is one of the most aggressive types of cancers and is difficult to cure by conventional procedures, it is necessary to identify novel molecular markers for the assessment of prognosis and as potential therapeutic targets. Moreover, there seems to be a paucity of the research concerned with the involvement of CD24 on gallbladder carcinoma. Hence, in this retrospective study, we evaluated the prognostic value of CD24 expression for gallbladder carcinoma.

Materials and Methods

Patients and Tissue Samples

The study was approved by the Research Ethics Committee of The Second Xiangya Hospital of Central South University, P.R.China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Specimens from 207 patients with gallbladder carcinoma who consecutively underwent surgery between January 2004 and May 2009 in the Second Xiangya Hospital of Central South University were included in the present study. All specimens had been collected, diagnosed, and stored by the Central Pathology Laboratory at the Department of Pathology, the Second Xiangya Hospital of Central South University. Samples were taken from 108 females and 99 males (aging 33~78 years, mean±SD=52.6±10.1 years). Gallbladder carcinoma was diagnosed on the basis of histological findings and was staged according to the TNM system (nodal status and depth of wall infiltration) according to the American Joint Committee on Cancer. The clinicopathological features of the patients are summarized in Table 1. In addition, 20 gallbladder tissue samples obtained from patients with cholelithiasis were also used for immunohistochemistry. 188 patients were given a follow-up ranging from two months to five years.

Immunohistochemical Staining and Assessment

For immunohistochemical staining, tissues were fixed in 10% buffered formalin and embedded in paraffin. Commercially available monoclonal antibody to CD24 (Santa Cruz Biotechnology, California) was used. Immunohistochemical staining was carried out on sections using the avidin-biotin method and a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). One paraffin-embedded block of tissues was selected from each patients and cut into 4μm sections. Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 10min before conducting antigen retrieval using a microwave oven at 95°C for 5 min and cooling at 25°C for 2 hours. After washing with PBS, blocking serum was applied for 10 min. The sections were incubated with anti-CD24 monoclonal antibody overnight at 4°C. After washing in PBS, a biotin-marked secondary antibody was applied for 10 min followed by a peroxidase-marked streptavidin for an additional 10min. The reaction was visualized by using 3, 3′-diaminobenzidine tetrahydrochloride. The nuclei were counterstained with hematoxylin. Positive and negative immunohistochemistry controls were routinely used. Reproducibility of staining was confirmed by reimmunostaining via the same method in multiple, randomly selected specimens.

Immunoreactivity was assessed by two investigators who were blinded to clinicopathologic data. Discrepancies were resolved by simultaneous reexamination of the slides by both investigators using a double-headed microscope. The criteria used for assessment were as previously reported [13]. Briefly, the staining intensity of CD24 was semiquantitatively scored as negative, weak, or moderate/strongly positive. A complete negative staining was scored as negative. A weak staining was defined as a minimal but unequivocal staining in less than 10% of tumor cells. Stronger or more extensive staining was scored as moderate/strong positive. For statistical analysis, patients with weak and moderate/strong staining were lumped together as a CD24 positive group, in comparison with those with CD24 negative tumors.

Statistical Analysis

SPSS13.0 software for Windows (SPSS Inc, USA) and SAS 9.1 (SAS Institute, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. Statistical analyses were performed with Fisher's exact test for any 2×2 tables, Pearson $\chi 2$ test for non- 2×2 tables, Kaplan-Meier method for the question of survival, Chiquest trend test for ordinal datum, Cox regression analysis for the multivariate analysis. The p values of less than 0.05 were considered to be statistically significant.

Table 1 Association between CD24 expression and conventional clinicopathological parameters in 207 patients with gallbladder carcinoma

Factor	NO.	CD24 expression (n, %)		p^a
		0	1	
Gender				
Male	99	20 (20.2)	79 (79.8)	0.58
Female	108	24 (22.2)	84 (77.8)	
Age at diagnosis				
≥70 years	137	29 (21.2)	108 (78.8)	0.56
<70 years	70	15 (21.4)	55 (78.6)	
Histological grade				
I	39	22 (56.4)	17 (43.6)	0.02
II	61	11 (18.0)	50 (72.0)	
III	107	11 (10.3)	96 (89.7)	
Histological type				
Adenocarcinoma	179	39 (22.8)	140 (78.2)	0.08
Squamous cell carcinoma	16	3 (18.7)	13 (81.3)	
Neuroendocrine carcinoma	12	2 (16.7)	10 (83.3)	
pT stage				
1	31	13 (41.9)	18 (58.1)	0.01
2	78	22 (28.2)	56 (71.8)	
3	72	7 (9.7)	65 (90.3)	
4	26	2 (7.7)	24 (92.3)	
Resection margin				
Negative	76	21 (27.6)	54 (72.4)	0.11
Positive	108	18 (16.7)	90 (83.3)	
Unknown[b]	23	5 (21.7)	18 (78.3)	

'[a]' X^2 test.

'[b]' Excluded from p calculations

Results

Immunohistochemical Detection of CD24 in Gallbladder Carcinomas

CD24 expression in the mucosal epithelium was negative in all 20 cases of cholelithiasis (Fig. 1a). Of 207 cases with gallbladder carcinoma, 163 (78.7%) showed CD24 positive expression in the mucosa. The staining pattern includes a membranous staining, cytoplasmic staining, or both (Fig. 1b~c). Generally, CD24 expression showed considerable heterogeneity in the intratumoral distribution.

Association Between CD24 Expression and the Clinicopathological Features of Gallbladder Carcinomas

The association of CD24 expression with the clinicopathological features of patients with gallbladder carcinoma was shown in Table 1. We did not find any significant

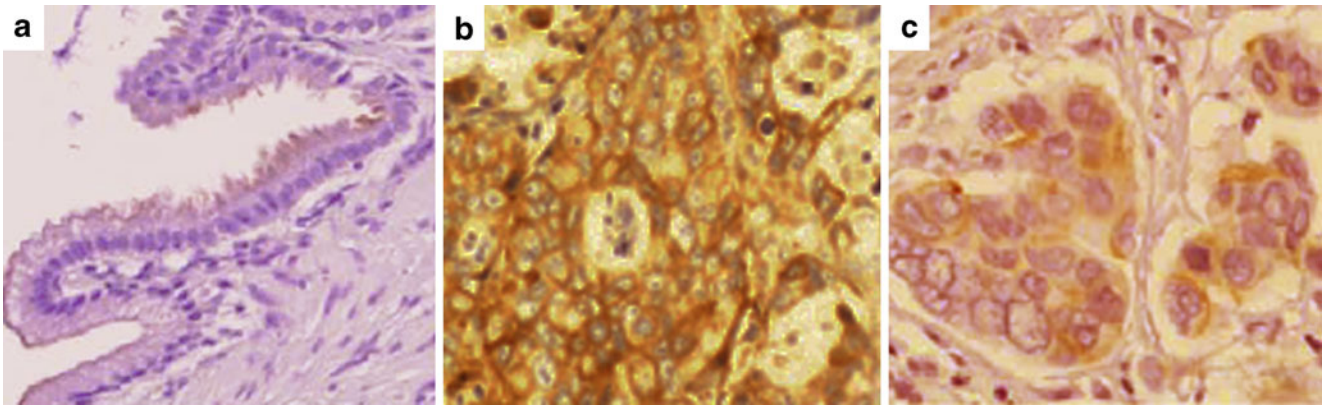

Fig. 1 Immunohistochemical staining for CD24 in gallbladder carcinoma (Original magnification ×200). **a**, CD24 staining was negative in non-tumor gallbladder tissues; **b**, CD24 expression was found in cell membrane at various levels in gallbladder carcinoma; **c**, CD24 staining was found in cytoplasm at various levels in gallbladder carcinoma

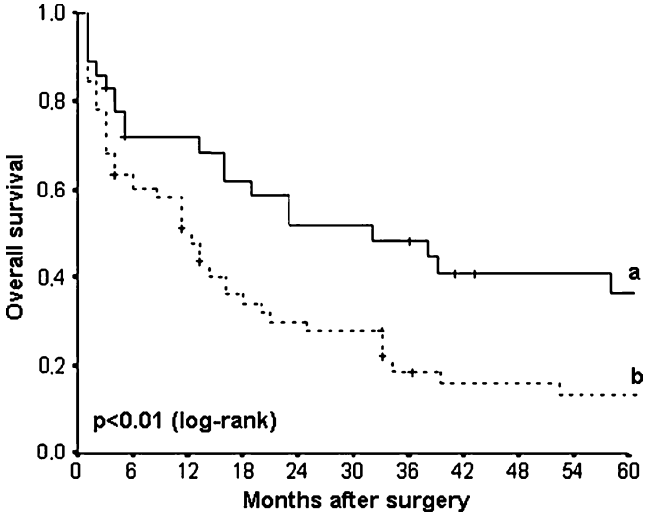

Fig. 2 Kaplan-Meier survival curves for CD24 expression in gallbladder carcinoma. 'a' categorized by negative CD24 expression; 'b', categorized by positive CD24 expression. Survival was significantly better for patients with CD24 - expression than those with positive expression ($p<0.01$)

association of CD24 staining with patients' gender and age, tumor histological type and resection margin involvement. The positive expression of CD24 tended to be significantly associated with tumor histological grades ($P=0.02$) and pT stages ($P=0.01$). Especially, the incidences of CD24 positive expression were significantly higher in gallbladder carcinomas with grade 3 and stage pT4 than those with lower grades and stages.

CD24 Expression Patterns and Prognosis of Patients with Gallbladder Carcinoma

188 patients were given a follow-up ranging from two months to five years and there are 75 patients had been still living by the end of the present research. The association between 5-year survival rate and the expression of CD24 was analyzed using Kaplan-Meier method.

As expected, pT stage ($P=0.01$), grade ($P=0.03$), and positive resection margins ($P=0.02$) were of prognostic value, whereas gender, patient age, and tumor histological type were prognostically not relevant. Of note, CD24 positive expression was associated with a poor prognosis (Fig. 2; $P<0.01$). The median survival times of patients presenting tumors with and without CD24 expression were 9 and 17 months, respectively. Accordingly, the estimated 5-year overall survival rates were 8.0% (15/188) and 31.9% (60/188). Multivariate analysis including pT stage, grade, resection margin involvement, and CD24 expression (Table 2) identified CD24 positive expression as an independent prognostic marker ($P=0.02$; relative risk, 1.6; 95% confidence interval, 1.2~3.6). Additionally, pT stage and the involvement of resection margins but not tumor grade were independent prognostic markers.

Discussion

This is the first report demonstrating an independent prognostic value of CD24 positive expression for gallbladder carcinoma. Similar results were obtained by Weichert et al. [14] in colorectal adenocarcinoma, Kristiansen et al. [11, 15] in ovarian carcinoma and breast cancer, Winkler et al. [16] in urothelial carcinoma and Kristiansen et al. [10] in non-small cell lung cancer. It remains to be seen whether in these carcinomas CD24 expression represents merely a surrogate marker for prognosis or whether it plays a pathogenic role for carcinogenesis and tumor progression. Although CD24 functions as a mucin-like adhesion molecule which enhances the metastatic potential of malignant-cells, it also has been described as a diagnostic molecular marker of malignant tumor and for patient prognosis. To date, however, final proof for a direct carcinogenic or tumor promoting role of CD24 expression is lacking.

The incidence of human gallbladder carcinoma is still too high, and its clinical courses are quite variable. It is of general importance to be able to predict the biology of this disease and thus, to predict its clinical course in the individual patient to ensure adequate treatment and patient monitoring. Conventional prognostic and predictive

Table 2 Multivariate analysis (Cox regression) of different prognostic parameters in patients with gallbladder carcinoma

	Overall survival		
	P	Relative risk	95% confidence interval
CD24 expression	0.02	1.6	1.2~3.6
Tumor grade	Not significant		
pT stage	0.01		
1 vs. 2	0.02	1.9	1.0~4.1
1 vs. 3	0.01	2.2	1.3~6.8
1 vs. 4	0.01	2.8	1.2~8.9
Resection margin	0.03	1.3	1.0~2.9

markers for gallbladder carcinoma are histological grade, tumor cell differentiation, and pT stage [17]. Moreover, molecular markers are being searched for and established to allow for a refined classification of prognosis, especially for the patient subgroups whose outcome can be only insufficiently predicted by the classical, previously established parameters. Among others, candidate genes of current interest are the various cell adhesion molecules, matrix metalloproteinases, cytokines and some growth factors [18, 19]. CD24 is another recently identified novel prognostic marker gene that is showing great promise. It is a heavily glycosylated phosphatidylinositol anchored cell-surface protein; physiologically, CD24 is expressed by pre-B lymphocytes, and it becomes lost during the maturation process to plasma cells [20]. In contrast to the mouse model, CD24 is not expressed on human erythrocytes or thymocytes [21]. It plays a crucial role in cell selection and maturation during hematopoiesis. CD24 expression is reported also during the embryonic period on developing neural cells and pancreatic cells [22]. It was shown to be expressed in various hematologic malignancies and in the solid tumors of some organs, and it was recently reported to be expressed in the tumors of ovary and breast [23]. CD24 is overexpressed in various malignant tissues, including B-cell lymphomas [24], gliomas [25], and small cell lung [10], hepatocellular [26], uterine [27], ovarian [15], breast [11], prostate [12], and pancreatic carcinomas [28]. Recent studies have reported increased expression of CD24 is usually tied with a more aggressive course of the disease. Because CD24 is expressed at the multistep process of carcinogenesis and associated with the clinical features (histological grades and pT stages) of gallbladder carcinoma, we do not surprise that it can predict prognosis, which is consistent with the previous studies on other tumors, such as breast cancer [11], ovarian cancer [15] and intrahepatic cholangiocarcinoma [13]. As we know, surgical resection is the only potentially curative therapy for gallbladder carcinoma. Unfortunately, most patients with this disease present with unresectable disease. Decisions regarding the extent of surgical management of patients with gallbladder carcinoma strongly depend on the pT status. Patients with potentially resectable pT2, pT3, or pT4 stage disease who underwent radical cholecystectomy showed a significantly better survival than patients treated with simple cholecystectomy [29, 30]. In this study, CD24 overexpression was significantly correlated with pT status. So, it would be tempting to suggest radical cholecystectomy for all operable CD24-overexpressing gallbladder carcinoma and dependent on the nodal status administer additional adjuvant radiation and systemic chemotherapy within prospectively randomized clinical trials.

According to the results of this study, CD24 may serve as an important target in chemoprevention for the following reasons. The first is that its increased expression occurs at an early stage of the multistep process of carcinogenesis in gallbladder carcinoma. Second, CD24 protein expression is strongly correlated with histological grade and pT stage of tumors. The third reason is CD24 may predict the prognosis of patients with gallbladder carcinoma.

In conclusion, our data demonstrate for the first time that CD24 is an important marker of malignancy and poor prognosis in gallbladder carcinoma. Its detection combined with cancerous staging may increase the ability of investigators to predict the prognosis of patients with gallbladder carcinoma. Furthermore, the CD24 antigen represents an attractive target for specific therapies with monoclonal antibodies in patients with CD24-overexpressing gallbladder carcinoma, so the detection of CD24 may help clinicians select patients likely to benefit from novel molecular therapies.

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