Clinicopathological significance of DNA fragmentation factor 45 and thyroid transcription factor 1 expression in benign and malignant lesions of the gallbladder

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Gallbladder cancer (GBC) is one of the most aggressive tumors; we examined the expression level of DNA fragmentation factor 45 (DFF45) and thyroid transcription factor 1 (TTF-1) in benign and malignant lesions of the gallbladder by immunohistochemistry. The results were correlated with clinicopathological features and prognosis. DNA fragmentation factor 45 and TTF-1 expression was significantly higher in gallbladder adenocarcinomas than in the corresponding peritumoral tissues ($\chi^2_{DFF45} = 6.92$, $\chi^2_{TTF\text{-}1} = 8.68$, ps < 0.01), polyps ($\chi^2_{DFF45} = 4.49$, $\chi^2_{TTF\text{-}1} = 5.35$, ps < 0.05), and chronic cholecystitis ($\chi^2_{DFF45} = 12.98$, $\chi^2_{TTF\text{-}1} = 17.74$, ps < 0.01). Negative expression of DFF45 and TTF-1 was significantly associated with tumor differentiation, tumor mass, lymph node metastasis and invasion of adenocarcinomas ($p < 0.05$). Univariate Kaplan-Meier analysis showed that elevated expression levels of DFF45 and TTF-1 ($p < 0.05$) were closely associated with increased overall survival. In addition, the average survival time of patients with DFF45(+) TTF-1(+) tumors was significantly higher than those with DFF45(–) TTF-1(–) tumors ($p < 0.05$). Finally, multivariate Cox regression analysis showed that negative expression of DFF45 and TTF-1 was an independent prognostic predictor in gallbladder adenocarcinoma ($p < 0.05$). The expression of DFF45 and/or TTF-1 is closely related to the carcinogenesis, progression, clinical behavior and prognosis of gallbladder adenocarcinomas. DNA fragmentation factor 45 and TTF-1 could be progression-associated genes correlating with good prognosis in GBC.

Key words: gallbladder neoplasms, gallbladder polyp, chronic cholecystitis, DNA fragmentation factor 45, thyroid transcription factor 1.

Introduction

Gallbladder cancer (GBC) is the fifth most common gastrointestinal cancer and the most common biliary tract malignancy in the USA [1-3]. The incidence of gallbladder cancer has undergone a marked increase in China [4]. The surgical removal of the gallbladder with part of the liver and lymph node dissection is currently the most common treatment for resectable GBC. However, GBC is detected at an early stage in only about 10% of patients, who are viable candidates for surgery. Most cases of GBC are discovered at an advanced, inoperable stage with serosal invasion and metastasis to other organs [5]. Generally, palliative chemotherapy

and radiation therapy offer almost no benefit in GBC. Patients with unresectable or metastatic GBC have an extremely poor prognosis and most of them die in less than one year following the diagnosis [1]. Thus, precise identification of the lithogenic and other genes that might have a role in GBC susceptibility should help identify individuals at increased risk among susceptible subpopulations [6]. Additionally, identifying functionally important and clinically relevant metastasis genes will provide possible targets for antimetastatic therapy and potentially improve GBC patient prognosis.

DNA fragmentation factor-45 (DFF45) is the substrate of caspase-3, a key point effector molecule in apoptosis, and thus has an important role in apoptotic DNA fragmentation, which contributes to malignant transformation and metastasis [7, 8]. Recent clinical studies have convincingly linked DFF45 with tumor progression and poor clinical outcomes in many cancer types, including neuroblastoma, esophageal carcinoma, ovarian endometriomas [9-11], and endometrial and ovarian carcinoma [10, 12-15]. However, the involvement of DFF45 in human GBC has not been reported.

Thyroid transcription factor 1 (TTF-1) is a tissue-specific transcription factor highly expressed in thyroid follicle cells and type II alveolar epithelial cells. The expression level of TTF-1 is significantly correlated with growth, development, malignant behavior and prognosis of thyroid and lung cancer [16-18]. In addition, recent studies have suggested that TTF-1 is significantly correlated with growth, development and malignant behavior of cervical small cell neuroendocrine carcinomas, ovarian cancer and some rare intracranial tumors [14, 19, 20].

In this study, we examined the expression of DFF45 and TTF-1 in benign and malignant lesions of the gallbladder by immunohistochemistry. To the best of our knowledge, this is the first study of DFF45 and TTF-1 expression in benign and malignant gallbladder lesions. In addition, the expression of DFF45 and TTF-1 was correlated with clinicopathologic characteristics, clinical behavior prognosis of adenocarcinomas, and patient survival.

Material and methods

Case selection

A total of 204 specimens (108 adenocarcinomas, 46 corresponding peritumoral tissues, 15 gallbladder polyps and 35 chronic cholecystitis tissues) were included in this study. All diagnoses were based on morphological criteria, immunohistochemical staining, and clinical findings. Surgery included radical resection for 34 adenocarcinomas (31.5%), palliative surgery for 48 adenocarcinomas (44.4%), and no operation with only surgical biopsy for 26 cases (24.1%). Detailed clinicopathological characteristics of the specimens are presented in Table I. Survival information for 67 patients with adenocarcinoma was obtained through letters and phone calls. Twenty patients (29.9%) survived over 1 year and 47 (70.1%) patients survived less than 1 year.

All experiments were performed in full agreement with ethics and international conventions. Ethics Committee approval was obtained from the Ethic Committee for Human Study of Central South University.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue samples. Rabbit anti-human DFF45/ICAD and TTF-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For visualization, a ChemMate™EnVison +/HRP/DAB staining kit was used according to the manufacturer's protocol. Briefly, the sections were deparaffinized and then incubated with peroxidase inhibitor (3% H_2O_2) in the dark for 10 minutes, followed by a EDTA-trypsin digestion for 15 minutes. The sections were incubated with primary antibody for 60 minutes at 37°C. Slides were then rinsed in PBS and incubated for 30 minutes at 37°C with solution A from the Dako ChemMate™ staining kit. 3,3'-Diaminobenzidine (DAB) substrate was added for 15 minutes followed by hematoxylin counter-staining. The slides were then dehydrated with different concentrations (70-100%) of ethanol, soaked in xylene for 15 minutes and mounted in neutral balsam.

The percentage of positive DFF45 or TTF-1 cells was calculated from 10 random microscopic fields. The sections with ≥ 25% positive cells were denoted as positive whereas the sections with less than 25% positive cells were denoted as negative.

Statistical analysis

Data were analyzed using SPSS 13.0. The correlation between DFF45 and TTF-1 expression with clinicopathological characteristics was analyzed using χ^2 independent tests. Fisher's exact probability test was used for analyzing associations between the two independent sample groups. Correlations were considered significant when the two-tailed p-value was less than 0.05. Kaplan-Meier and log-rank tests were used for univariate survival analysis. The Cox proportional hazards model was used for multivariate analyses and to determine the 95% confidence interval (95% CI).

Results

DFF45 and TTF-1 expression in benign and malignant lesions of gallbladder

Immunohistochemical analysis revealed that DFF45 positive staining was mainly localized in the cytoplasm

Table I. Specimens used in the study and corresponding patient clinicopathological characteristics

SPECIMEN TYPE AND CLINICOPATHOLOGICAL CHARACTERISTICS	
Adenocarcinoma (n)	**108**
age (mean ± SD), years	35-70 (52.6 ±11.2)
male, n (%)	31 (28.7)
female, n (%)	77 (71.3)
invasion into gallbladder surrounding tissues and organs, n (%)	59 (54.6)
regional lymph node metastasis, n (%)	59 (54.6)
presence of gallstones, n (%)	58 (53.7)
histopathologic subtypes, n (%)	
adenoma canceration	9 (8.2)
well-differentiated adenocarcinoma	29 (26.9%)
moderately differentiated adenocarcinoma	29 (26.9%)
poorly differentiated adenocarcinoma	30 (27.8)
mucinous adenocarcinoma	11 (10.2)
Peritumoral tissue (distance from the tumor ≥ 3 mm) (n)	**46**
normal, n (%)	10 (21.7)
mild dysplasia, n (%)	10 (21.7)
moderate dysplasia, n (%)	12 (26.1)
severe dysplasia, n (%)	14 (30.5)
Gallbladder polyps (φ = 8-15 mm) (n)	**15**
male, n (%)	5 (33.3)
female, n (%)	10 (66.7)
normal to mild dysplasia, n (%)	10 (66.7)
moderate to severe dysplasia, n (%)	5 (33.3)
Chronic cholecystitis (n)	**35**
chronic cholecystitis, n (%)	15 (42.9)
chronic cholecystitis accompanied by gallbladder stone, n (%)	20 (57.1)
normal gallbladder mucosa	11 (31.4)
mild dysplasia	12 (34.3)
moderate dysplasia	7 (20.0)
severe dysplasia	5 (14.3)

and/or the cell nucleus (Fig. 2A and 2B), while TTF-1 positive staining was mainly localized in the nucleus and/or cytoplasm (Fig. 2C and 2D). The expression of DFF45 and TTF-1 was significantly higher in gallbladder adenocarcinoma than in the corresponding adjacent peritumoral tissues, adenomatous polyps and chronic cholecystitis epithelium (all $p < 0.05$) (Table II).

DFF45 and TTF-1 expression and clinicopathological characteristics of gallbladder adenocarcinomas

As shown in Table III, the positive immunohistochemical reaction for DFF45 and TTF-1 was significantly more frequent in well-differentiated adenocarcinomas with no lymph node metastasis and no invasion, as compared to the poorly differentiated adenocarcinomas with lymph node metastasis and invasion to the gallbladder surrounding tissues and organs (all $p < 0.05$). DNA fragmentation factor 45 and TTF-1 exhibited no significant association with mucinous adenocarcinoma or other clinicopathological characteristics, such as sex, age, and history of gallstones ($p > 0.05$).

The relationship between DFF45 and TTF-1 expression in gallbladder adenocarcinomas

Of the 45 DFF45 positive adenocarcinomas, TTF-1 was positively expressed in 38 cases, while of the 63 DFF45 negative adenocarcinomas, TTF-1 was negatively expressed in 13 cases ($\chi^2 = 32.82$, $p < 0.01$), suggesting a high consistency between the expression of DFF45 and TTF-1 in gallbladder adenocarcinomas.

DFF45 and TTF-1 expression and survival of patients with gallbladder adenocarcinoma

In this study, the survival information was available for 67 of the 108 patients with gallbladder adenocarcinoma. Positive DFF45 and TTF-1 immunohistochemical expression was present in 27/67 (40.3%) and 32/67 (47.8%) of the analyzed samples, respectively.

The Kaplan-Meier survival analysis revealed that the histological type ($p = 0.031$), tumor size ($p = 0.003$), lymph node metastasis ($p = 0.005$), invasion ($p = = 0.002$) and operative procedure ($p < 0.001$) were significantly associated with the average survival time of patients. The average survival time of patients with DFF45 and TTF-1 positive specimens was significantly

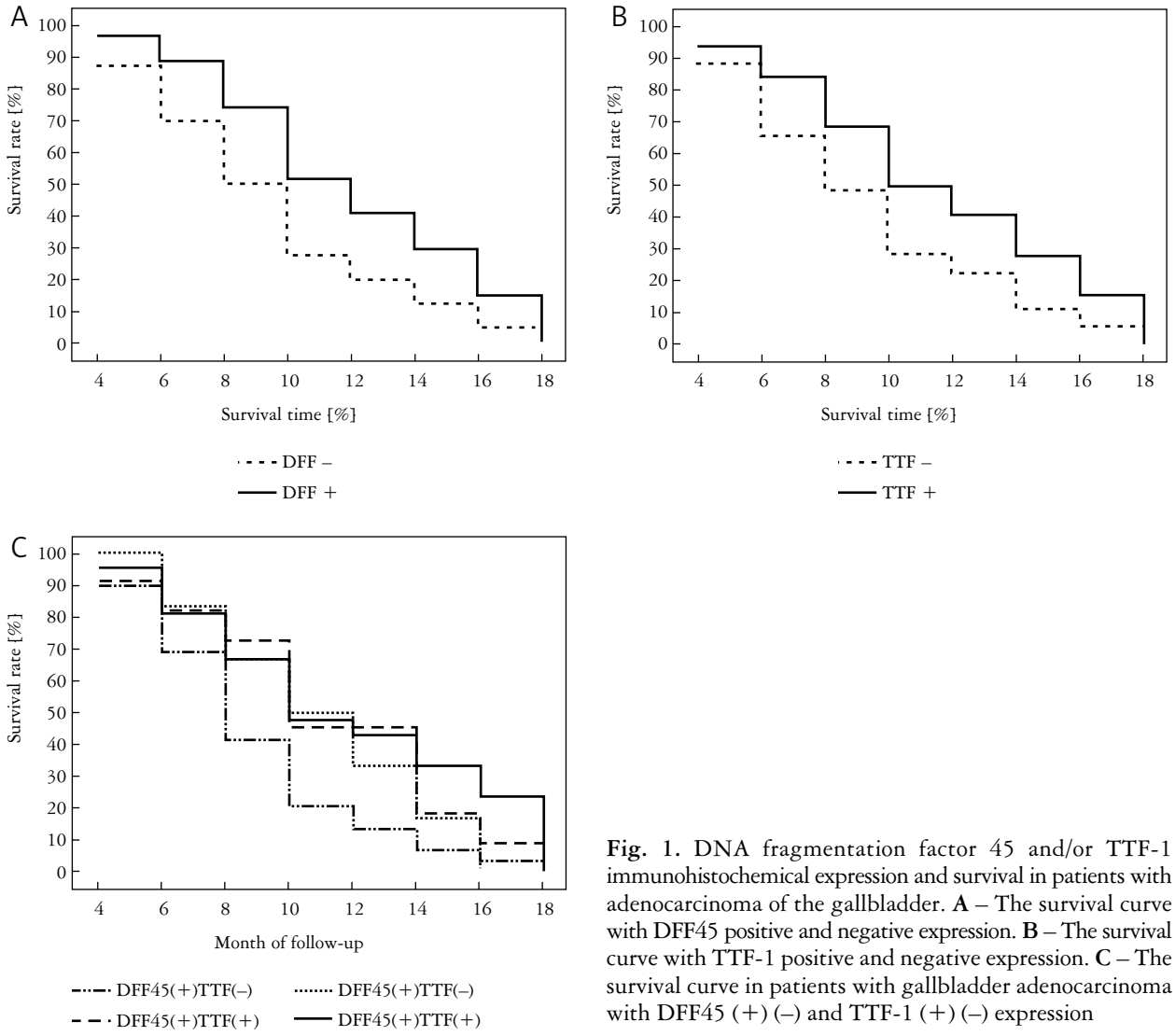

Fig. 1. DNA fragmentation factor 45 and/or TTF-1 immunohistochemical expression and survival in patients with adenocarcinoma of the gallbladder. **A** – The survival curve with DFF45 positive and negative expression. **B** – The survival curve with TTF-1 positive and negative expression. **C** – The survival curve in patients with gallbladder adenocarcinoma with DFF45 (+) (–) and TTF-1 (+) (–) expression

higher than those with negative expression ($p = 0.025$ and $p = 0.042$, respectively) (Table IV, Fig. 1A and 1B). Cox multivariate analysis showed that the tumor size (≥ 2 cm), lymph node metastasis, invasion, as well as DFF45 and TTF-1 negative expression negatively correlated with postoperative survival, yet positively correlated with mortality, suggesting that DFF45 and TTF-1 are relative risk factors on prognosis in GBC (Table V).

Discussion

The ability of tumor cells to evade apoptosis is one of the key hallmarks of cancer [21]. Several apoptotic pathways have been described, identifying caspase-3 and caspase-7 as key effector molecules in apoptosis [22, 23]. DNA fragmentation factor 45 is a caspase-3 or caspase-7 substrate that must be cleaved before apoptotic DNA fragmentation can proceed.

Table II. The expression of DFF45 and TTF-1 in the benign and malignant lesions of the gallbladder

TISSUE TYPE (NUMBER OF SAMPLES)	DFF45			TTF-1		
	POSITIVE N (%)	χ^2	P*	POSITIVE N (%)	χ^2	P*
gallbladder adenocarcinoma (108)	45 (41.7)			56 (51.7)		
peritumoral tissue (46)	9 (19.6)	6.92	< 0.01	12 (26.1)	8.68	< 0.01
polyp (15)	2 (13.3)	4.49	< 0.05	3 (20.0)	5.35	< 0.05
chronic cholecystitis (35)	3 (8.6)	12.98	< 0.01	4 (11.4)	17.74	< 0.01

**DFF45 and TTF-1 expression in a specific tissue compared to the expression in gallbladder adenocarcinoma*

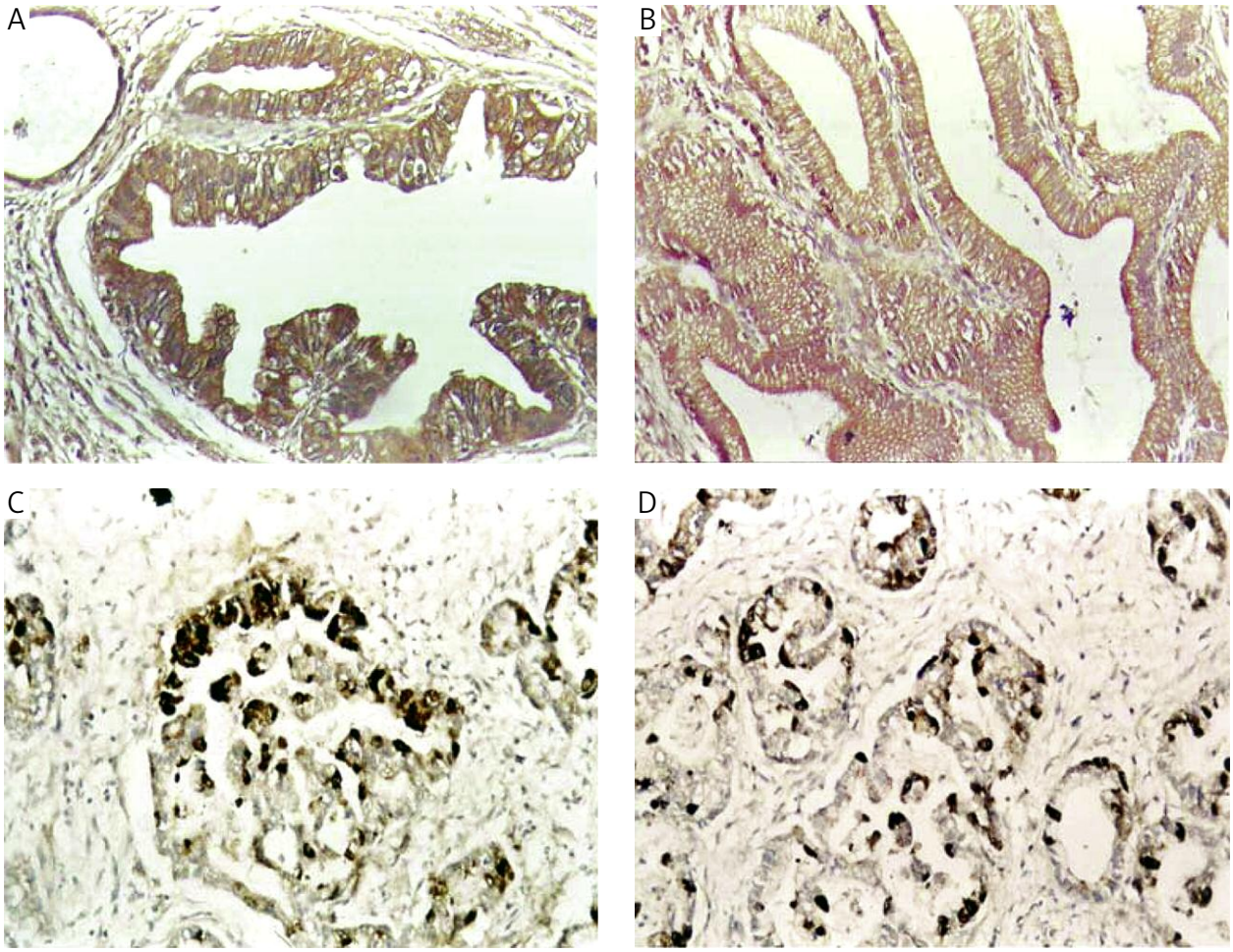

Fig. 2. DNA fragmentation factor 45 and TTF-1 expression in the benign and malignant lesions of the gallbladder. DFF45 positive immunohistochemical reaction was mainly localized in the cytoplasm and TTF-1 positive immunohistochemical reaction was mostly localized in the nucleus. **A** – DFF45 positive expression in a well-differentiated adenocarcinoma. **B** – DFF45 positive expression in an atypical hyperplasia of gallbladder epithelium in chronic cholecystitis. **C** – TTF-1 positive expression in a moderately differentiated adenocarcinoma. **D** – TTF-1 positive expression in a moderately atypical hyperplasia of gallbladder epithelium in polyp (original magnification 200×)

DNA fragmentation factor 45 forms a heterodimer with DFF40, a 40 kDa endonuclease, and acts both as its specific inhibitor and as a chaperone in its appropriate folding [7, 8]. When caspase-3 is activated by apoptotic stimuli, it cleaves DFF45 at two sites, which causes the release and activation of DFF40, leading to the generation of double-stranded breaks in internucleosomal chromatin regions and chromatin condensation [11].

Thus, aberrant DFF45 expression might influence tumor development and progression and DFF45 may play a role in malignant transformation and metastasis. Indeed, DFF45 expression was down-regulated or lost in neuroblastoma, endometrial carcinoma, ovarian carcinoma, esophageal carcinoma, gastroenteric carcinoma, bladder carcinoma and germinoma. In addition, in some tumor types, DFF45 expression correlated with tumor progression, differentiation, metastasis and prognosis. Taken together, these findings support a tumor suppressor role of DFF45 [8, 10, 12, 13, 15, 24].

Tissue-specific transcription factors are important regulators of cell determination and differentiation. Thyroid transcription factor 1, also known as NKX-2.1, is a tissue-specific transcription factor expressed in the thyroid and lung [25]. Thyroid transcription factor 1 binds to the thyroglobulin and thyroid peroxidase gene promoters and regulates the expression of thyroid-specific genes, and thus has an important role in thyroid growth and development. In addition, TTF-1 is highly expressed in type II alveolar epithelial cells and is essential for lung morphogenesis and differentiation. Recent findings show that TTF-1 expression level is significantly correlated with growth, development, malignant behavior and prognosis of thyroid carcinoma and non-small-cell lung cancer (NSCLC). Tumors with high TTF-1 expression are well differentiated, progress slowly with almost no lymph node metastasis, and usually have a good prognosis [16, 18, 24]. In addition, many studies show that TTF-1 expression is significantly correlated with growth, development, malignant be-

Table III. The association of DFF45 and TTF-1 expression with the clinicopathological characteristics of gallbladder adenocarcinoma

CLINICOPATHOLOGICAL CHARACTERISTIC	CASES (N)	DFF45			TTF-1		
		POSITIVE N (%)	χ^2	P	POSITIVE N (%)	χ^2	P
Sex							
male	24	11 (45.8)	0.22	> 0.05	12 (50.0)		
female	84	34 (40.5)			44 (52.3)	0.04	> 0.05
Age (year)							
≤ 45	31	12 (38.7)	0.17	> 0.05	13 (41.9)	1.71	> 0.05
>45	77	33 (42.9)			43 (55.8)		
Histopathologic subtypes*							
well-differentiated	36	21 (58.3)	8.43	< 0.05	26 (72.2)	11.90	< 0.05
moderately differentiated	31	12 (38.7)			15 (48.4)		
poorly differentiated	30	7 (23.3)			9 (30.0)		
mucinous	11	5 (45.5)			6 (54.5)		
Maximum diameter of tumor (cm)							
< 2	31	16 (51.6)	1.77	> 0.05	20 (64.5)	2.79	> 0.05
≥ 2	77	29 (37.6)			36 (46.8)		
Lymph node metastasis							
(–)	49	27 (55.1)	6.66	< 0.01	32 (65.3)	6.50	< 0.05
(+)	59	18 (30.5)			24 (40.7)		
Locoregional invasion							
(–)	49	26 (53.1)	4.79	< 0.05	31 (63.3)	4.68	< 0.05
(+)	59	19 (32.2)			25 (42.3)		
Cholecystolithiasis							
(–)	50	20 (40.0)	0.11	> 0.05	24 (48.0)	0.55	> 0.05
(+)	58	25 (43.1)			32 (55.2)		

**Comparison of well-differentiated and poorly differentiated adenocarcinoma: 2 DFF45 = 8.2, P < 0.01; 2 TTF-1 = 11.71, P < 0.01*

havior and prognosis of other tumors, such as cervical small cell neuroendocrine carcinomas, ovarian cancer and some rare intracranial tumors [14, 19, 20].

In our study we examined the expression of DFF45 and TTF-1 in benign and malignant gallbladder lesions by immunohistochemistry. We found that DFF45 and TTF-1 expression was significantly higher in well-differentiated adenocarcinomas without lymph node metastasis and locoregional invasion compared to poorly differentiated adenocarcinomas with lymph node metastasis. Moreover, patients with tumors with higher DFF45 and TTF-1 expression had better prognosis. Since the cleavage of DFF45 by caspase-3 is an important step in the apoptotic pathway, down-regulation or loss of DFF45 expression in neoplastic tissues might result in the blockage of the caspase-3 mediated apoptotic pathway and consequently lead to tumor progression. Thus, our findings are in agreement with the tumor suppressor role of DFF45 [4, 11, 26].

In addition, DFF45 and TTF-1 protein expression in gallbladder adenocarcinomas was significantly higher than that in the corresponding peritumoral tissues, adenomatous polyps or chronic cholecystitis epithelium. Furthermore, DFF45 and/or TTF-1 expression was significantly higher in well-differentiated adenocarcinomas < 2 cm in diameter without lymph node metastasis and locoregional invasion. The survival time was longer in patients with tumors positive for DFF45 and TTF-1 than those with negative expression, as shown by the Kaplan-Meier survival analysis.

Another interesting finding of this study was that DFF45 and TTF-1 expression levels significantly correlated with each other in gallbladder adenocarcinomas, which may be related to their respective biological functions.

The results of the Cox multivariate analysis suggest that positive expression of DFF45 and TTF-1 positively correlated with the postoperative survival rate and negatively correlated with the death rate. In addition, DFF45 and TTF-1 were found to be independent prognostic factors for gallbladder adenocarcinomas.

Finally, the results of our study suggest that the expression levels of DFF45 and/or TTF-1 are closely related to the carcinogenesis, clinical behavior, and prognosis of gallbladder adenocarcinoma. Based on our results we can conclude that the expression of DFF45 and TTF-1 could be used as new potential biomarkers and/or targets for GBC therapy.

The authors declare no conflict of interest.

Table IV. Relationship between DFF45 and TTF-1 expression, clinicopathological characteristics and average survival of gallbladder adenocarcinoma patients

CLINICOPATHOLOGICAL CHARACTERISTICS	SAMPLES (N)	AVERAGE SURVIVAL (MONTHS)	P
Sex			
male	19	10.0 (4-16)	0.910
female	48	10.0 (4-18)	
Age			
≤ 45	11	8.0 (4-14)	0.121
>45	56	10.0 (4-18)	
Histopathologic subtypes			
adenoma canceration	8	12.0 (8-18)	
well-differentiated	20	10.0 (4-18)	
moderately differentiated	20	10.0 (4-18)	0.031
poorly differentiated	12	8.0 (4-10)	
mucinous	7	10.0 (6-16)	
Maximum diameter of tumor (cm)			
<2 cm	20	14.0 (4-18)	0.003
≥ 2 cm	47	8.0 (4-18)	
Lymph node metastasis			
(–)	36	12.0 (4-18)	0.005
(+)	31	8.0 (4-18)	
Locoregional invasion			
(–)	39	10.0 (4-18)	0.002
(+)	28	8.0 (4-16)	
DFF45 expression			
+	27	11.9 (4-18)	0.025
–	40	9.4 (4-18)	
TTF-1 expression			
+	32	11.6 (4-18)	0.042
–	35	9.4 (4-18)	
DFF45 and TTF-1 expression			
DFF45(+)TTF-1(+)	21	11.8 (4-18)	
DFF45(+)TTF-1(–)	6	1.0 (4-16)	0.049
DFF45(–)TTF-1(+)	11	11.3 (4-18)	
DFF45(–)TTF-1(–)	29	8.9 (4-18)	

Table V. Multivariate Cox regression analysis of survival rate in 67 gallbladder adenocarcinoma patients

GROUP FACTOR	REGRESSION COEFFICIENT	STANDARD ERROR	RELATIVE RISK	P	95% CI	
					LOWER LIMIT	UPPER LIMIT
Pathology type adenoma, well-/moderately/poorly differentiated, mucinous adenocarcinoma	0.558	0.293	1.75	0.089	0.98	3.10
tumor diameter < 2.0/≥ 2.0 cm	0.899	0.336	2.46	0.039	1.27	4.75
metastasis of lymph node n/y	1.087	0.399	2.97	0.020	1.36	6.48
invasiveness of regional tissues n/y	1.236	0.421	3.44	0.017	1.51	7.86
Resection procedure: radical/non-radical	1.403	0.513	4.07	0.005	1.49	11.12
DFF45 –/+	–0.703	0.321	0.50	0.031	0.26	0.93
TTF-1 –/+	–0.611	0.290	0.54	0.047	0.31	0.96

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