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RESEARCH

Expression of Cell Cycle-Related Proteins, p16, p53 and p63 as Important Prognostic Markers in Gallbladder Adenocarcinoma

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Abstract Gallbladder cancer, the most common biliary tract malignancy, is a highly malignant neoplasm. In the present work, we have analyzed the significance of cell cycle-related proteins to predict prognosis and to provide guidance for optimal therapeutic decision-making in patients with gallbladder adenocarcinoma. The expressions of p16, p21, p27, p53, p63, cyclin D1, bcl-2 and bcl-6 were examined in a tissue microarray constructed from 96 cases of gallbladder adenocarcinoma by immunohistochemistry and correlated with clinicopathologic prognostic factors. Expression of p16 was correlated with a low pT stage, adenoma background and good prognosis. Cases with p63 expression showed a higher T stage, more frequent perineural invasion and poor prognosis when compared to cases without p63 expression. Over-expression of p53 or loss of p53 was associated with poor tumor differentiation, frequent distant metastasis and low disease-specific survival rate. The expressions of p21, p27, bcl-2, bcl-6 and cyclin D1 were not significant prognostic factors for gallbladder adenocarcinoma. These results indicate that p16, p63 and p53 can be used as prognostic markers in gallbladder adenocarcinoma; especially p53 and p63 as poor prognostic markers and p16 as a favorable prognostic marker.

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Introduction

Gallbladder cancer is the most common biliary tract cancer and the fifth most common cancer of the digestive system [1]. It has a distinctly higher incidence in certain demographic groups and geographic areas, and Korea belongs to a high risk group [1]. Despite enormous improvements in diagnosis and surgical techniques, the prognosis of gallbladder cancer generally remains poor with the 5-year survival for all stages of gallbladder cancer being approximately 5 %. Surgery is an effective treatment option for patients with resectable gallbladder cancers. For patients with unresectable gallbladder cancers, palliative chemotherapy and radiotherapy remain the only possible treatment options; however, they provide minimal survival benefit [2]. Hence, attempts to identify molecular prognostic markers and candidates for targeted therapy are needed, although the conventional clinical prognostic factors in gallbladder cancers such as histological grade, depth of wall infiltration and lymph node metastasis have already been defined.

Similar to other neoplasms, gallbladder cancer is also considered to develop due to the accumulation of multiple genetic alterations [3]. Among them, disruption of cell cycle control is a pathognomonic feature of malignant cells that can manifest as dysregulation of cyclin protein expression, enhanced activity of cyclin-dependent kinases, altered expression or function of cyclin-dependent kinase inhibitors and mutation of cell cycle check-point controls [4]. Although many studies have been performed to demonstrate cell cycle

regulatory proteins as prognostic markers in gallbladder cancers, the results remain controversial.

Herein, we examined the immunohistochemical expressions of cell cycle regulatory proteins including p16, p21, p27, p53, p63, cyclin D1, bcl-2 and bcl-6 in 96 cases of gallbladder adenocarcinoma and then correlated the results with the clinicopathologic findings to determine the prognostic significance of cell cycle regulatory proteins in gallbladder adenocarcinoma.

Materials and Methods

Patients and Clinical Data

This study was approved by the Institutional Review Board of Kangbuk Samsung Hospital. This retrospective study included 96 consecutive cases of gallbladder adenocarcinoma that underwent cholecystectomy, which was performed at Kangbuk Samsung Hospital from December 1990 to March 2011. Patients' clinical information including age, sex, the day of initial diagnosis, follow-up and radiological findings was obtained from electronic medical records. The information about patient deaths was obtained from the medical record department of Kangbuk Samsung Hospital and National Cancer Information Center.

Microscopic Evaluation of Cholecystectomy Specimens

All the cases were microscopically reviewed for determination of histological classification according to the 2010 World Health Organization Tumor Classification [5]. The tumor differentiation, depth of wall invasion, tumor location, gross tumor configuration, resection margin status, lymphovascular invasion, perineural invasion, and concurrent dysplasia/adenoma were also evaluated. The tumor stage was assigned according to the 2010 AJCC Tumor Node Metastasis (TNM) staging system [6].

Construction of Tissue Microarray

Tissue microarray blocks (2-mm-diameter cores) were manually generated from the formalin-fixed, paraffin-embedded gallbladder adenocarcinoma tissues. Two representative cores were obtained from each tumor block for tissue microarray blocks. One core of normal tonsil and one core of placenta were included in each tissue microarray block as positive controls.

Immunohistochemical Staining

The primary antibodies used in this study are summarized in Table 1. Immunohistochemical staining was performed automatically using an autostainer (BOND-MAX, Leica, Newcastle, United Kingdom). Antigen retrieval was performed by steaming with EDTA (pH 9.0) for 20 min using a microwave. Negative controls included the omission of the primary antibodies.

The expression of the proteins except for p53 was recorded as the percentage of positive cells with moderate to strong staining intensity. In case of p53, three expression patterns were recorded: 1) homogeneously strong positive expression (strong nuclear staining in more than 90 % of tumor cells), 2)

Table 1 Antibodies for Immunohistochemical staining

Antibody	Product	Dilution
p16	$p16^{INK4a}$, mtm laboratories, Heidelberg, Germany	1:2
p21	p21, Leica, Newcastle, United Kingdom	1:50
p27	p27, Novocastra, Newcastle, United Kingdom	1:50
P53	p53, Dako, Glostrup, Denmark	1:5000
p63	p63, Zeta, Arcadia, CA, USA	1:200
cyclin D1	cyclin D1, Labvision, Fremont, CA, USA	1:100
bcl-2	BCL2, Dako, Glostrup, Denmark	1:100
bcl-6	BCL6, Novocastra, Newcastle, United Kingdom	1:200

Table 2 Clinicopathologic features in 96 cases of gallbladder adenocarcinoma

Variables		Number of cases (%)
Differentiation	Well	41 (42.7)
	Moderate	41 (42.7)
	Poor	14 (14.6)
pT stage	1a	24 (25)
	1b	8 (8.3)
	2	41 (42.7)
	3	23 (24)
Lymphovascular invasion	Absence	87 (90.6)
	Presence	9 (9.4)
Perineural invasion	Absence	75 (78.1)
	Presence	21 (21.9)
Lymph node metastasis[a]	Absence	29 (58)
	Presence	21 (42)
Distant metastasis	Absence	64 (66.7)
	Presence	32 (33.3)
Intraepithelial neoplasia[b]	Absence	23 (27.7)
	Low grade	13 (15.7)
	Intermediate grade	6 (7.2)
	High grade	41 (49.4)
Adenoma background	Absence	64 (66.7)
	Presence	32 (33.3)

[a] Lymph node dissection is performed in 50 cases

[b] Thirteen cases are excluded due to severe erosion of remaining mucosa

heterogeneous expression (nuclear staining with variable intensity in more than 10 % of tumor cells), and 3) negative expression (weak nuclear staining in less than 10 % of tumor cells).

Statistical Analysis

The data were analyzed by PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) software. Crosstabs, Pearson's chi-square test, Fisher's exact test, Kruskal-Wallis test and logistic regression test were used as needed. The Kaplan-Meier and Cox regression tests were employed to analyze the survival data. Differences were regarded as statistically significant at p-values $<.05$.

Results

Clinicopathologic Features

The clinicopathological data of 96 gallbladder adenocarcinoma cases are summarized in Table 2. The mean age of patients was 65.7 years (range; 29 to 89 years, median; 68 years). The most frequent tumor location was the body (35 cases, 36.4 %), followed by the fundus (29 cases, 30.2 %), and the neck with cystic duct (8 cases, 8.4 %). Some tumors extended into the entire gallbladder, fundus along with body, and body along with neck in 17 cases (17.7 %), 5 cases (5.2 %) and 2 cases (2.1 %), respectively. The tumors exhibited variable gross configurations; flat/infiltrative (42 cases, 43.7 %), polypoid (28 cases, 29.2 %), papillary (14 cases, 14.6 %) and nodular (12 cases, 12.5 %). Most of the tumors showed well or moderate differentiation. Three (3.1 %) tumors showed a focal sarcomatous component. Thirty-two cases of tumor (33.3 %) occurred in adenoma, which were associated with better tumor differentiation ($p<.001$), lower T stage ($p<.001$) and favorable prognosis ($p<.001$). The resection margin could be evaluated in 93 cases (96.9 %) and it was involved by the tumor in 26 cases (27.9 %). The tumor has recurred in 4 cases (4.2 %) of which in 3 cases, the resection margin was involved by the tumor.

The mean follow-up period was 36.7 months (range; 0.2 to 231.4 months) in 96 cases. Among 32 patients with distant metastasis, nine patients (28.1 %) presented with metastatic

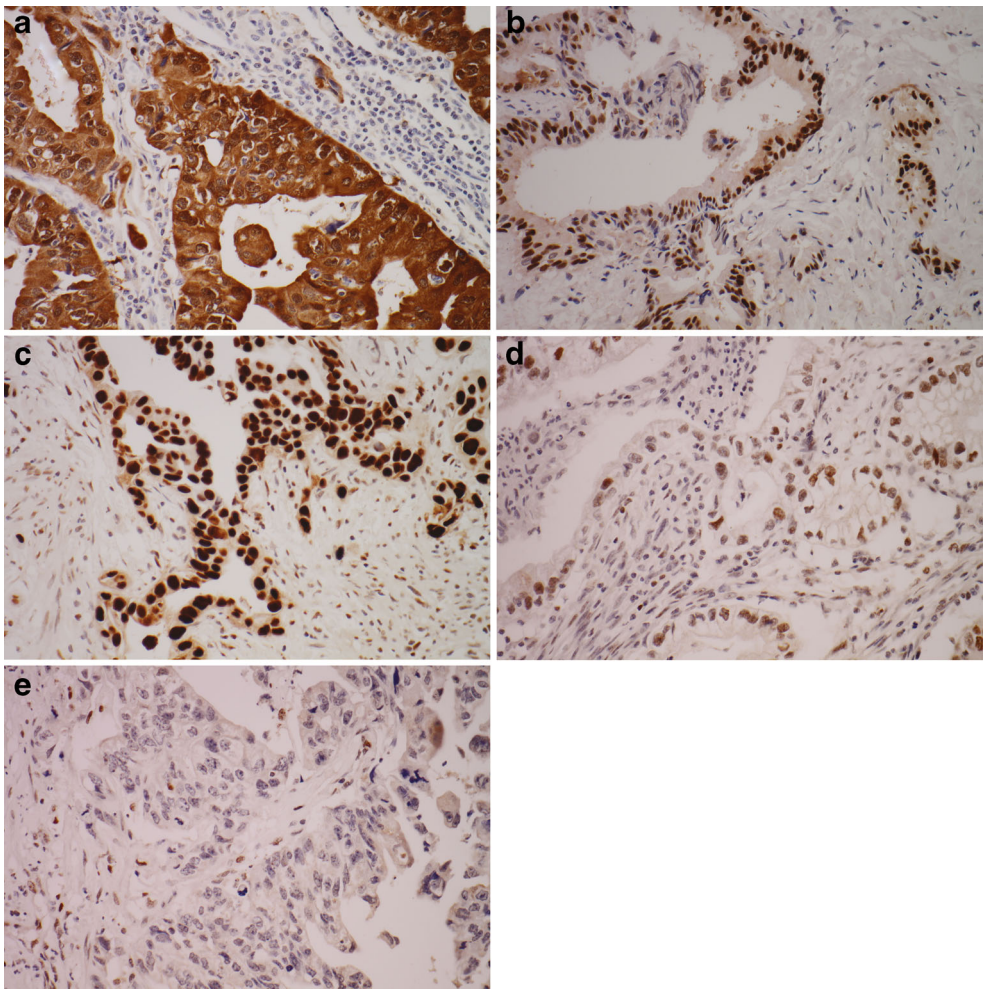

Fig. 1 Immunohistochemical staining for p16 (**a**), p63 (**b**) and p53 (**c**–**e**). p16 stains in the nuclei and cytoplasm (**a**). p63 is expressed in the nuclei (**b**). Three expression patterns of p53: homogeneously strong expression (**c**), heterogeneous expression with variable staining intensity (**d**), and negative expression (**e**)

adenocarcinoma at the time of initial diagnosis and in the remaining 23 patients, metastasis developed at a mean 10.6 months post-operatively (range; 0.7 to 56.4 months). The most common metastatic sites were liver (17 patients), followed by peritoneum (10 patients), non-regional lymph nodes (6 patients), lungs (4 patients), bone (3 patients), abdominal wall (2 patients) and adrenal glands (1 patient). At the time of the last follow-up, 46 patients (47.9 %) were dead, of which 43 patients (44.8 %) died because of gallbladder adenocarcinoma.

Immunohistochemical Staining Results and Clinicopathologic Parameters

Immunohistochemical analysis revealed staining of p21, p27, p53, p63, cyclin D1, bcl-2 and bcl-6 in the nuclei, and p16 staining was observed not only in the nuclei but also in the cytoplasm (Fig. 1). The staining intensity and pattern of two tumor cores were similar.

p16 expression was observed in 41 cases (range; 5–100 %). Among them, 39 cases were interpreted as positive for p16 expression with a 10 % cut-off value. p16 positivity was correlated with favorable prognosis, lower pT stage ($p=.047$), frequent adenoma background ($p=.004$), and better disease-specific patient survival ($p<.001$) than negative p16 expression (Table 3 and Fig. 2a). The p63 staining was observed in 27 cases (range; 1–99 %); and in seven cases, p63 staining was considered positive with a 10 % cut-off value. p63 positivity was associated with higher pT stage ($p=.007$), more frequent perineural invasion ($p=.047$) and more frequent biliary intraepithelial neoplasia ($p=.034$) (Table 3). Patients with p63-positive gallbladder adenocarcinoma showed a rapid decrease in survival rate within the first post-operative year (Fig. 2b). For p53, the tumor showed homogeneously strong positivity in 38 cases (45.2 %) and negativity in 19 cases (22.6 %). The remaining cases revealed heterogeneous nuclear staining similar to that in the adjacent non-neoplastic epithelium. On dividing the cases into two groups of 1) homogeneously strong positive or negative expression, and 2) heterogeneously positive expression, the former group showed poorer tumor differentiation ($p=.009$), more frequent distant metastasis ($p=.011$) and poorer disease-specific patient survival than the latter group (Table 3 and Fig. 2c). When we divided the cases into three groups based on p53 expression, two groups with over-expression of p53 and loss of p53, respectively showed lower disease-specific survival rate than the group with heterogeneous expression of p53 with a similar survival graph pattern (Fig. 2d).

Univariate analysis revealed high pT stage, poor tumor differentiation, presence of perineural invasion, presence of lymphovascular invasion, p63 expression, and strong positivity

Table 3 Correlation between expression of p16, p63 and p53 proteins and prognostic factors in 96 cases of gallbladder adenocarcinoma

Variables		p16			p63			p53		
		Negative $n=52$ (57.1 %)	Positive $n=39$ (42.9 %)	p	Positive $n=84$ (92.3 %)	Negative $n=7$ (7.7 %)	p	Heterogeneous expression $n=27$ (32.1 %)	Over-expression or loss $n=57$ (67.9 %)	p
Differentiation	Well	20 (38.5)	19 (48.7)	.205	38 (45.2)	1 (14.3)	.255	18 (66.7)	21 (36.8)	**.009**
	Moderate	22 (42.3)	16 (41)		34 (40.5)	4 (57.1)		8 (29.6)	27 (47.4)	
	Poor	10 (19.2)	4 (10.3)		12 (14.3)	2 (28.6)		1 (3.7)	9 (15.8)	
pT stage	1a&1b	13 (25)	18 (46.2)	**.047**	31 (36.9)	0 (0)	**.007**	13 (48.2)	18 (31.6)	.177
	2	24 (46.2)	14 (35.9)		36 (42.9)	2 (28.6)		9 (33.3)	24 (42.1)	
	3	15 (28.8)	7 (17.9)		17 (20.2)	5 (71.4)		5 (18.5)	15 (26.3)	
Lymph node metastasis[a]	Absence	16 (50)	12 (80)	.063	27 (61.4)	1 (33.3)	.557	6 (75)	22 (64.7)	.697
	Presence	16 (50)	3 (20)		17 (38.6)	2 (66.7)		2 (25)	12 (35.3)	
Distant metastasis	Absence	32 (61.5)	30 (76.9)	.172	57 (67.9)	4 (57.1)	.680	24 (88.9)	35 (61.4)	**.011**
	Presence	20 (38.5)	9 (23.1)		27 (32.1)	3 (42.9)		3 (11.1)	22 (38.6)	
Lymphovascular invasion	Absence	47 (90.4)	35 (89.7)	>.999	75 (89.3)	7 (100)	>.999	23 (85.2)	52 (91.2)	.460
	Presence	5 (9.6)	4 (10.3)		9 (10.7)	0 (0)		4 (14.8)	5 (8.8)	
Perineural invasion	Absence	41 (78.8)	29 (74.4)	.625	67 (79.8)	3 (42.9)	**.047**	23 (85.2)	42 (73.7)	.278
	Presence	11 (21.2)	10 (25.6)		17 (20.2)	4 (57.1)		4 (14.8)	15 (26.3)	
Biliary intraepithelial neoplasia[b]	Absence	10 (22.7)	11 (31.4)	.447	34 (46.6)	0 (0)	**.034**	10 (38.5)	9 (18.7)	.094
	Presence	34 (77.3)	24 (68.6)		39 (53.4)	6 (100)		16 (61.5)	39 (81.3)	
Adenoma background	Absence	41 (78.8)	19 (48.7)	**.004**	54 (64.3)	6 (85.7)	.415	13 (48.1)	40 (70.2)	.058
	Presence	11 (21.2)	20 (51.3)		30 (35.7)	1 (14.3)		14 (51.9)	17 (29.8)	

[a] Lymph node dissection are performed in 50 patients

[b] Thirteen cases with severe mucosal erosion are excluded

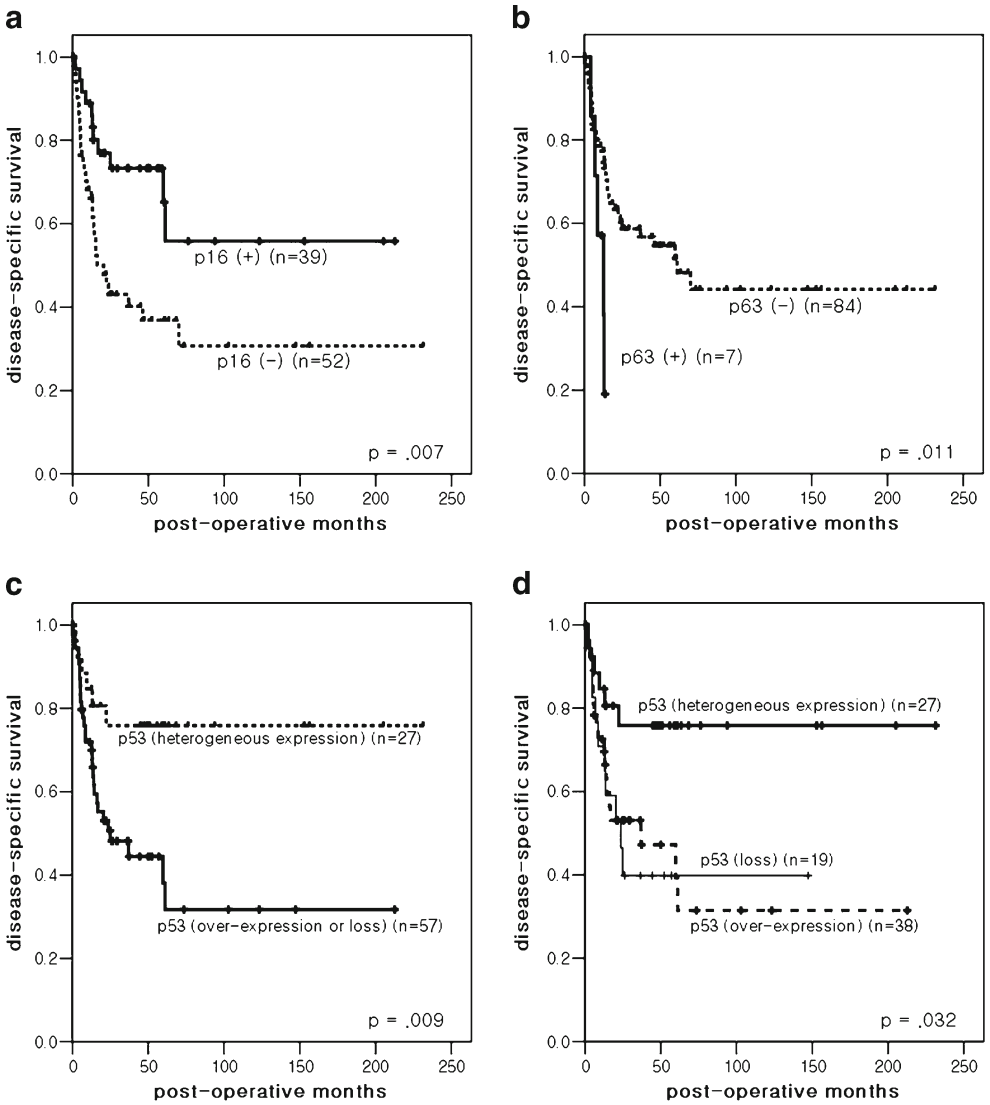

Fig. 2 Disease-specific survival curves according to expression of p16 (**a**), p63 (**b**) and p53 proteins (**c** and **d**) by Kaplan-Meier survival analysis. Cases with p16 expression show favorable prognosis (**a**). Cases with p63 expression reveal a rapid decrease in survival rate within the first post-operative year (**b**). The over-expression of p53 or loss of p53 expression is associated with a poor patient survival rate (**c**). On dividing the cases into three groups based on p53 expression, two groups with over-expression of p53 and loss of p53, respectively exhibit a similar decrease in survival (**d**)

or loss of p53 as poor prognostic factors (Table 4); whereas, adenoma background and p16 expression were found to be favorable prognostic factors. On multivariate analysis considering pT stage, tumor differentiation, expression of p16, p53 and p63; pT stage was found to be the only significant prognostic factor ($p = .002$).

The other proteins, p21, p27, bcl-2, bcl-6 and cyclin D1, were expressed in 56 (62.2 %), 69 (77.5 %), 7 (7.7 %), 34 (38.2 %), and 39 (43.3 %) cases with a 10 % cut-off value, respectively. They did not show any significant correlation with clinicopathologic parameters and patient disease-specific survival.

Discussion

Uncontrolled cell growth and proliferation are the hallmarks of cancer, which occur mainly due to loss of control at the cell cycle check points [7]. Cell cycle is controlled by a family of cyclins, cyclin dependent kinases (CDKs) and their inhibitors (CDKIs) through activation and inactivation of phosphorylation events [8]. These cell cycle-related proteins are not only known as markers for cancer but also as prognostic factors for various diseases.

The p16 protein is a tumor suppressor gene protein, which is a CDK inhibitor that regulates the G1-S phase of the cell cycle. The loss of p16 expression has been known as a poor prognostic factor, and it is correlated with tumor progression or decreased patient survival in lung and pancreatic cancer [9, 10], However, contradictory results have been reported about the contribution of p16 expression in gallbladder carcinogenesis [3, 11–13]. Some researchers have reported that nuclear p16 expression was rare in normal gallbladder epithelium and it was a frequent event in high-grade dysplasia and adenocarcinoma, suggesting that p16 over-expression is an early and relatively common event in carcinogenesis of the gallbladder [11, 12, 14].

Table 4 Univariate and multivariate analysis for disease-specific death

Variables		Univariate analysis				Multivariate analysis			
		HR	95.0 % CI		*p*	HR	95.0 % CI		*p*
			Lower	Upper			Lower	Upper	
pT stage	1a or 1b	1			.001				.002
	2 or 3	71.427	5.863	870.159		72.391	4.605	1138.086	
Differentiation	Well	1			<.001				
	Moderate	3.623	1.667	7.873					
	Poor	6.909	2.847	16.765					
Perineural invasion	Absence	1			<.001				
	Presence	3.933	2.018	7.667					
Lymphovascular invasion	Absence	1			.027				
	Presence	2.692	1.118	6.483					
Adenoma background	Presence	1			<.001				
	Absence	7.862	2.761	22.381					
p16	Positive	1			.009				
	Negative	2.520	1.261	5.036					
p63	Negative	1			.017				
	Positive	3.338	1.245	8.949					
p63	Heterogeneous expression	1			.013				
	Over-expression or loss	3.067	1.265	7.431					

However, others reported that p16 immunoreactivity was more frequently found in normal gallbladder epithelium than in dysplasia, adenoma or carcinoma of the gallbladder [3, 13]. In our study, among 8 cores of normal gallbladder mucosa, only one core showed focal p16 positivity and the adenoma component also rarely showed p16 positivity. However, we could not assess p16 expression in biliary intraepithelial neoplasia and carcinoma *in situ*. To better understand gallbladder carcinogenesis, more studies with gallbladder precancerous lesions should be performed in future.

With respect to the prognostic significance of p16 in gallbladder adenocarcinoma, p16 expression was correlated with low pT stage, frequent adenoma background and good prognosis in the present study, suggesting that loss of p16 expression could be a poor prognostic factor in gallbladder adenocarcinoma. Two previous studies have shown different results; 1) Shi *et al*. reported that loss of p16 protein was not associated with any clinicopathologic factors or patient survival, and 2) Tadokoro *et al*. also reported that there was no significant correlation between p16 protein immunoreactivity and clinicopathologic parameters [15, 16]. However, Shi *et al*. and Tadokoro *et al*. enrolled only 37 and 51 cases of gallbladder carcinoma, respectively; and they included various histologic types of gallbladder carcinoma in their studies such as adenocarcinoma, adenosquamous carcinoma and undifferentiated carcinoma [15, 16].

The p53 mutation is an early event in gallbladder carcinogenesis and is associated with the metaplasia-dysplasia-carcinoma sequence of gallbladder cancer progression [17]. However, the prognostic significance of p53 expression has remained controversial in gallbladder cancer. Some researchers have reported that p53 expression is associated with poor prognostic parameters, but others have reported contradictory results [17–27]. In this study, over-expression of p53 protein or loss of p53 protein was correlated with poor disease-specific prognosis (Fig. 2c and d), thereby suggesting p53 as a candidate marker for poor prognosis in gallbladder adenocarcinoma. We included a larger number of gallbladder cancer cases than those in majority of prior studies and targeted only gallbladder adenocarcinoma cases, whereas previous studies included cases of various histologic types of gallbladder carcinoma such as adenocarcinoma, squamous cell, adenosquamous carcinoma, small cell and undifferentiated carcinoma [18–27]. In addition, we analyzed p53 expression in more details by considering staining intensity and percentage of stained tumor cells, whereas prior studies estimated p53 expression by a semi-quantitative method [18–27]. These differences between previous reports and our study may be the reason for the different results in both p16 and p53 expression.

The p63 protein has been recognized as a member of the p53 family. In cases with extrahepatic bile duct carcinoma, it has

been reported that p63 was overexpressed in 26.3 % of cases and it was associated with more frequent vascular invasion; however, there was no difference in survival between p63-expressing and p63-non-expressing tumors [28]. In another report, p63 overexpression was observed in 100 % of cholangiocarcinoma cases [29]. Frequency of p63 expression and its prognostic significance have not been clearly determined in gallbladder adenocarcinoma. In this study, only 7 cases (7.7 %) were found to be positive for p63 expression with a 10 % cut-off value, which were pure cases of gallbladder adenocarcinoma without squamous differentiation, and p63 expression was associated with poor prognosis, thereby suggesting p63 as a candidate marker for poor prognosis.

In summary, this study demonstrates that p16, p63 and p53 expression can be used as prognostic markers in gallbladder adenocarcinoma; especially p16 expression as a favorable prognostic marker, p63 expression as a poor prognostic marker, and over-expression of p53 or loss of p53 as a poor prognostic marker. Further studies including more number of gallbladder cancer cases are needed to accurately predict patient survival rate and to provide a guideline for making an optimal therapeutic decision.

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