Expression of the 14-3-3 sigma Protein and Methylation Status of the 14-3-3 sigma gene in Biliary Neoplasms

Dong Eun Song · Se Jin Jang
Jung-Sun Kim · Sang Soo Lee[1]
Myung-Hwan Kim[1] · Seung-Gyu Lee[2]
Young-Joo Lee[2] · Hae Joung Park[3]
Yhong-Hee Shim[3] · Eunsil Yu

Departments of Pathology, [1]Internal Medicine and [2]Surgery, University of Ulsan College of Medicine, Asan Medical Center, [3]Department of Biological Sciences and Bio/Molecular Informatics Center, Konkuk University, Seoul, Korea

Corresponding Author
Eunsil Yu, M.D.
Department of Pathology, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-dong, Songpa-gu, Seoul 138-736, Korea
Tel: 02-3010-4552
Fax: 02-472-7898
E-mail: esyu@amc.seoul.kr

Background : The 14-3-3 sigma (σ) protein has a negative regulatory role in the cell cycle progression of the. Down-regulation or overexpression of the 14-3-3σ protein has been reported in various human cancers. **Methods :** Immunohistochemistry for the 14-3-3σ protein was performed in non-neoplastic bile duct cells, intraductal papillary neoplasms of the liver (IPNL), mass-forming intrahepatic cholangiocarcinomas (ICC) and non-papillary extrahepatic cholangiocarcinomas (ECC). We investigated the methylation status of the 14-3-3σ gene in 45 cases of these 3 tumor groups. **Results :** The non-neoplastic bile duct cells demonstrated negative or weakly positive cytoplasmic immunoreactivity for the 14-3-3σ protein and no methylation of the 14-3-3σ gene. Overexpression as well as negative immunoreactivity associated with hypermethylation of the 14-3-3σ protein was observed in 16 (69.6%) of 23 cases of IPNL, in 21 (63.6%) of 33 cases of mass-forming ICC and in 27 (71.1%) of 38 cases of non-papillary ECC. Negative immunoreactivity was increased in the invasive IPNL (4/6, 66.7%), as well as in the poorly differentiated cases of mass-forming ICC (8/12, 66.7%) and the non-papillary ECC (5/8, 62.5%). **Conclusions :** The similar rates for the abnormal expression of the 14-3-3σ protein among the three groups of biliary neoplasms indicate its general association with biliary carcinogenesis. Furthermore, the loss of the 14-3-3σ protein may be involved in the tumor progression and differentiation in the biliary carcinogenesis.

Key Words : 14-3-3 proteins; Bile ducts; Biliary tract neoplasms; Immunohistochemistry; Methylation

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancers of adults.[1] It is classified macroscopically into mass-forming type, periductal infiltrative type and intraductal papillary type.[1] Intraductal papillary neoplasms of the liver (IPNL) are commonly designated as biliary papillomatosis, and they show a broad spectrum of cellular atypia from low grade dysplasia to invasive carcinoma in the proliferating biliary epithelial cells.[2] Dysregulation of the cell cycle has been suggested as a possible mechanism for the pathogenesis of IPNL.[3,4] Overexpression of p53 has also been demonstrated along with the histologic progression of IPNL.[3] In addition, IPNL have been shown to display frequent K-ras gene mutation, the same as in ICC.[4] However, IPNL were reported to be included in the CpG islands methylator phenotype negative group, which is in contrast to the other types of ICC.[5] In cases of mass-forming ICC, abnormal expressions of the G1-S modulators such as p53, cyclin D1, p16, p27, Rb, K-ras and p57,[6–8] and TGF-β/Smad

pathway are reported.[6] Lee *et al*. reported 59.5% CpG islands hypermethylation of the 14-3-3 sigma (σ) gene in ICC,[5] however, the expression level of the 14-3-3σ protein has not been reported. In cases of extrahepatic cholangiocarcinoma (ECC), decreased expression of p57[8] and hypermethylation of p16[9] were reported.

The 14-3-3 protein is one of the cell cycle regulatory proteins that comprises a family of highly conserved eukaryotic 25-33 kDa acidic polypeptides. Its σ isoform was originally isolated as an epithelial-specific marker and its expression was increased during epithelial differentiation.[10] The 14-3-3σ protein was also termed stratifin as a transformation-sensitive epithelial marker.[11] There are many evidences supporting the regulatory roles of the 14-3-3σ protein in the cell proliferation, differentiation, cell death and the signal transduction pathway.[11,12] For the progression of the cell cycle, the 14-3-3σ protein has a negative regulatory role for the G2/M checkpoint via sequestering the cdc2/

cyclinB1 complexes and the cdc25C in the cytoplasm.[13,14] It also inhibits G1/S progression by binding to cdk2 and cdk4, and the 14-3-3σ protein has emerged as a new class of cdk inhibitor.[14] Thus, the loss of its function may contribute to malignant transformation by impairing the G2/M and/or G1/S checkpoint functions.

Down-regulation of the 14-3-3σ protein expression was reported in various human cancers arising in the breast, the stomach, the colon, the lung, the liver, the urinary bladder, the vulva, the skin and lymphoid cells.[15-23] Inactivation of the 14-3-3σ gene was associated with hypermethylation of the 5′ CpG islands.[15,18,19,21-23] In contrast, overexpression of the 14-3-3σ protein was reported in papillary and anaplastic carcinomas of the thyroid gland,[24] pancreatic ductal adenocarcinomas[25,26] and ovarian cancers.[27,28]

Although the expression patterns of the 14-3-3σ protein in normal human epithelial cells have been variously reported according to the tissue types,[17] 14-3-3σ expression has not been described in the non-neoplastic intrahepatic and extrahepatic bile ducts cells. Furthermore, the expression pattern of the 14-3-3σ protein and the methylation status of the 14-3-3σ gene have not yet been reported in IPNL and ECC.

In the present study, the expression of the 14-3-3σ protein in the non-neoplastic intrahepatic and extrahepatic bile ducts cells was compared with that in the biliary neoplasms. We examined the expression of the 14-3-3σ protein in the subtypes of biliary neoplasms, including IPNL, mass-forming ICC and non-papillary ECC, to elucidate the implication of the 14-3-3σ protein expression in biliary carcinogenesis. In addition, the methylation status of the 5′ CpG islands of the 14-3-3σ gene was correlated with the expression of the 14-3-3σ protein to reveal the mechanism of the abnormal expression of the 14-3-3σ protein.

MATERIALS AND METHODS

Case selection

We selected 94 surgically resected specimens of biliary neoplasms, and they included 23 cases of IPNL, 33 cases of mass-forming ICC and 38 cases of non-papillary ECC (Table 1). They were obtained from the surgical pathology files of the Asan Medical Center from May 1996 to April 2003. Two pathologists reviewed the hematoxylin and eosin stained slides.

The twenty-three cases of IPNL were classified into 5 subtypes according to the degree of the cytologic and structural atypia, as was previously described.[2] Type 1 was defined as low grade dysplasia, type 2 high grade dysplasia, type 3 carcinoma in situ, type 4 carcinoma in situ with focal microscopic foci of stromal invasion and type 5 carcinoma with definite invasion into the hepatic parenchyma or into the fibromuscular layer of the large bile ducts. The thirty-three cases of mass-forming ICC and 38 cases of non-papillary ECC were classified into well-differentiated (WD), moderately differentiated (MD) and poorly differentiated (PD) tumors according to the degree of glandular differentiation of the tumor cells.

Immunohistochemistry

The formalin-fixed paraffin-embedded tissue blocks were cut into 4 to 6 μm thick sections. The sections were mounted on poly-L-lysine-coated glass slides, deparaffinized in zylene, rehydrated in graded alcohol and washed in tap water. The slides were incubated with 3% H_2O_2 and heated in a steam cooker with 10 mmol sodium citrate buffer, pH 6.0. After treatment with 10% normal goat serum for 10 min, mouse monoclonal antibody for 14-3-3σ (clone 1433S01, dilution 1:50; Neo Markers, Fremont, CA, USA) was applied for 1 h. After reacting with a biotinylated anti-mouse antibody for 10 min, the antigen-antibody complexes were visualized by using a streptavidin-horseradish peroxidase conjugate (LSAB kit; DAKO, Carpinteria, CA), with diaminobenzidine being used as a chromogen. The slides were counterstained with Meyer's hematoxylin. Sections of the normal skin were used for a positive control. The primary antibody was replaced with *H. pylori* monoclonal antibody for a negative control. The immunoreactivity was determined according to the staining intensity and the proportion of positive cells. When the tumor cells showed stronger immunoreactivity for the 14-3-3σ

Table 1. Cases examined in this study

Pathologic Dx (Case No.)	Subtype (Case No.)
IPNL (23)	Type 1 (4)
	Type 2 (2)
	Type 3 (5)
	Type 4 (6)
	Type 5 (6)
Mass-forming ICC (33)	WD (3)
	MD (18)
	PD (12)
Non-papillary ECC (38)	WD (6)
	MD (24)
	PD (8)

Dx, diagnosis; IPNL, intraductal papillary neoplasms of the liver; ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

protein than did the normal intrahepatic and extrahepatic bile ducts cells, the cells were considered to be positive. The immunoreactivity was defined as negative when the positive cells were $\leq 1/3$ of the tumor cells, and the immunoreactivity was defined as positive when the positive cells were >1/3 of the tumor cells. Immunolocalization of the 14-3-3σ protein was evaluated as being either cytoplasmic or membranous.

Analysis of the methylation status of the 14-3-3σ gene

We examined the methylation status of the 14-3-3σ gene promoter in 45 specimens, including 15 cases of IPNL, 14 cases of mass-forming ICC and 16 cases of non-papillary ECC. For the normal control, non-neoplastic intrahepatic and common bile ducts cells were microdissected with using a laser capture microdissection technique. The neoplastic areas on the hematoxylin and eosin-stained slides were matched for the corresponding 10 to 20 μm thick paraffin sections, and then they were scraped. The collected materials were deparaffinized by washing them in xylene and then rinsing them in ethanol. After the samples were digested with proteinase K, the DNA was extracted with using phenol/chloroform and it was precipitated with ethanol. The extracted DNA was modified by sodium bisulfite as previously described by Herman et al.[29] Methylation specific PCR (MSP) for the 14-3-3σ gene was performed with the following condition: 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension step for 10 min at 72°C. The reaction mixture contained 50 ng of modified DNA, 10 pmol of primers, 0.2 mM dNTP and 1 unit of Taq polymerase (Takara, Kyoto, Japan) in $1 \times$ PCR buffer (10 mM Tris (pH 8.3), 50 mM KCl and 1.5 mM MgCl$_2$). The primer sequences were as follows: (methylated band, forward) 5′-TGGTAGTTTTTATGAAAGGCGTC-3′; (methylated band, reverse) 5′-CCTCTAACCGCCCACCACG-3′; (unmethylated band, forward) 5′-ATGGTAGTTTTTATGAAAGGTGTT-3′; (unmethylated band, reverse) 5′-CCCTCTAACCACCCACCACA-3′. The PCR products were analyzed on 2.5% agarose gel, stained with ethidium bromide and visualized by UV illumination.

Statistical analysis

Statistical analysis was performed using SPSS version 10.0 (SPSS, Inc., Chicago). The χ^2 test and Fisher's exact probability test were used for analysis of the results. p-values of less than 0.05 were considered to be significant.

RESULTS

Expression of the 14-3-3σ protein in the normal bile duct cells

Most of the non-neoplastic intrahepatic and common bile duct cells were negative for the 14-3-3σ protein. However, the hyperplastic intrahepatic bile duct cells (Fig. 1A) and the peribiliary glands of the common bile duct (Fig. 1B) were either negative or weakly positive for the 14-3-3σ protein only in the cytoplasm.

Expression of the 14-3-3σ protein in the IPNL, the mass-forming ICC and the non-papillary ECC

Thirteen (56.5%) of 23 cases of IPNL were positive for the 14-3-3σ protein (Table 2). Ten (76.9%) of 13 cases with 14-3-3σ-immunopositivity showed the unusual membranous staining pattern with or without cytoplasmic immunoreactivity (Fig. 1C). One (25.0%) of 4 cases of the type 1 IPNL showed positive immunoreactivity for the 14-3-3σ protein. Ten (76.9%) of 13 cases of the type 2, type 3 and type 4 IPNL showed positive immunoreactivity for the 14-3-3σ protein. In contrast, four (66.7%) of six cases of the type 5 IPNL were negative for the 14-3-3σ protein (Fig. 1D).

Sixteen (48.5%) of 33 cases of mass-forming ICC were positive for the 14-3-3σ protein (Table 2). Two (66.7%) of three cases of WD mass-forming ICC were positive, while eight (66.7%) of 12 cases of PD mass-forming ICC were negative for the 14-3-3σ protein (Fig. 1E). However, there was no statistical significance in the immunoreactivity according to the degree of the histologic differentiation. Eleven (68.8%) of 16 cases of mass-forming ICC with immunopositivity for the 14-3-3σ protein showed membranous staining (Fig. 1F) with or without cytoplasmic immunoreactivity (Fig. 1G). There was no significant difference in the staining pattern among the WD, MD and PD mass-forming ICC (p=0.845).

Twenty-five (65.8%) of 38 cases of ECC were positive for the 14-3-3σ protein (Table 2). The rate of positive immunoreactivity was much lower in the PD ECC (3/8, 37.5%, Fig. 1H) than in the WD (6/6, 100.0%) and MD (16/24, 66.7%) ECC. The rate of positive immunoreactivity was statistically significant between the WD and PD ECC (p=0.031). Fifteen (60.0%) of 25 cases of ECC with positive immunoreactivity for the 14-3-3σ protein showed membranous staining (Fig. 1I) with or without cytoplasmic immunoreactivity (Fig. 1J). There was no signifi-
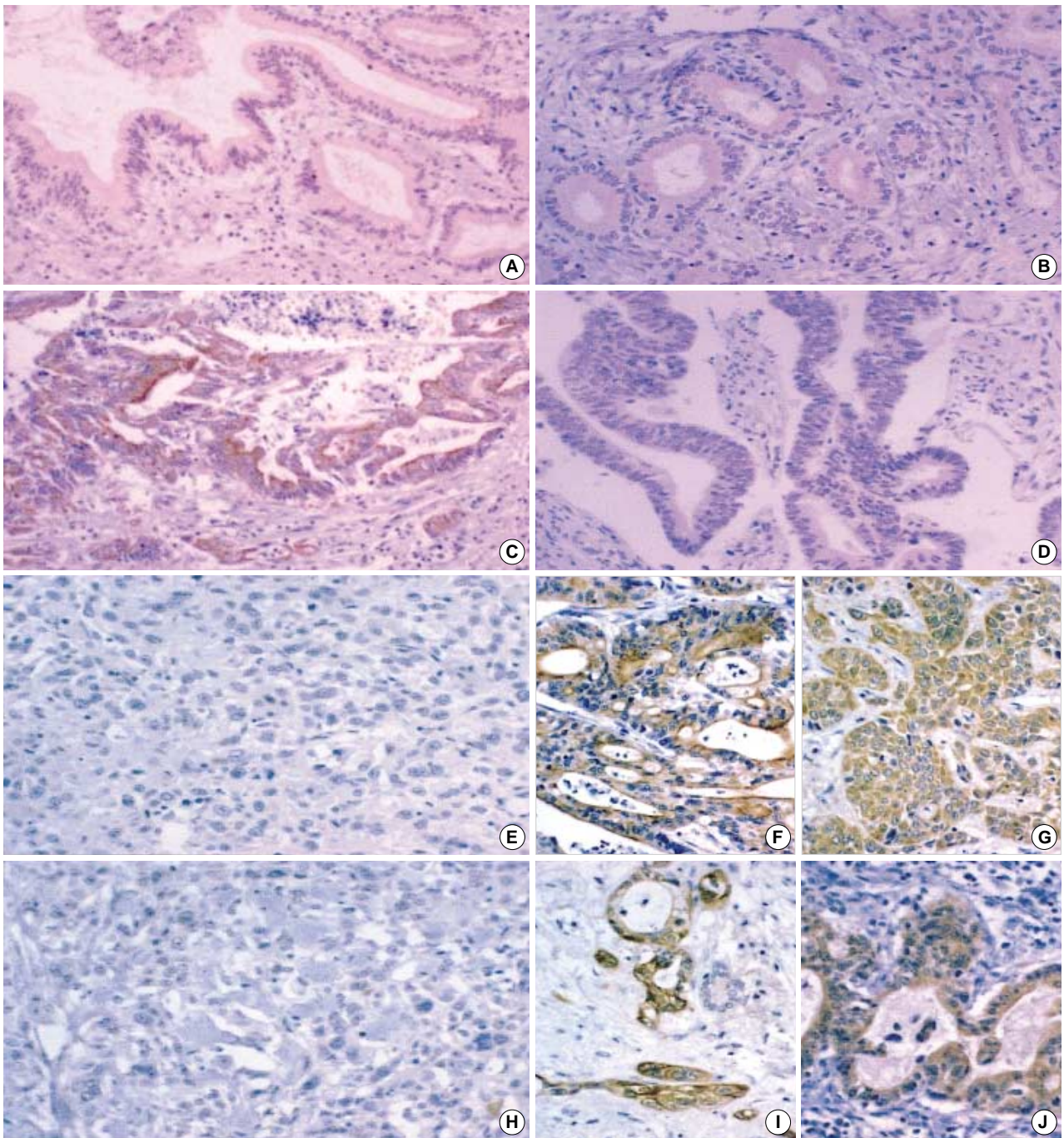
Fig. 1. Immunohistochemical results of the 14-3-3σ protein in bile duct cells and biliary neoplasms. (A) Hyperplastic intrahepatic bile duct cells and (B) peribiliary glands of common bile duct are either negative or weakly positive for the 14-3-3σ protein only in cytoplasm. Cases of type 5 IPNL demonstrate positive membranous (C) or negative (D) immunoreactivities for the 14-3-3σ protein. (E) A case of poorly differentiated (PD) mass-forming ICC is totally negative for the 14-3-3σ protein. Cases of moderately differentiated (MD) mass-forming ICC demonstrate membranous (F) or cytoplasmic (G) immunoreactivities. (H) A case of PD ECC is totally negative for the 14-3-3σ protein. Cases of MD ECC demonstrate membranous (I) or cytoplasmic (J) immunoreactivities.

cant difference in the staining pattern among the WD, MD and PD ECC (p=0.492).

Methylation status of the 14-3-3σ gene

Methylation of the 14-3-3σ gene was not found by MSP in

Table 2. Results of immunohistochemical stains for the 14-3-3σ protein in biliary neoplasms

Pathologic Dx (No.)	Immunoreactivity case No. (%)		Staining pattern case No. (%)	
	Negative	Positive	Cytoplasmic	Membranous with/without cytoplasmic
IPNL (23)	10 (43.5)	13 (56.5)	3 (23.1)	10 (76.9)
Type 1 (4)	3 (75.0)	1 (25.0)	0 (0.0)	1 (100.0)
Type 2 (2)	0 (0.0)	2 (100.0)	1 (50.0)	1 (50.0)
Type 3 (5)	2 (40.0)	3 (60.0)	0 (0.0)	3 (100.0)
Type 4 (6)	1 (16.7)	5 (83.3)	2 (40.0)	3 (60.0)
Type 5 (6)	4 (66.7)	2 (33.3)	0 (0.0)	2 (100.0)
Mass-forming ICC (33)	17 (51.5)	16 (48.5)	5 (31.2)	11 (68.8)
WD (3)	1 (33.3)	2 (66.7)	0 (0.0)	2 (100.0)
MD (18)	8 (44.4)	10 (55.6)	4 (40.0)	6 (60.0)
PD (12)	8 (66.7)	4 (33.3)	1 (25.0)	3 (75.0)
Non-papillary ECC (38)	13 (34.2)	25 (65.8)	10 (40.0)	15 (60.0)
WD* (6)	0 (0.0)	6 (100.0)	1 (16.7)	5 (83.3)
MD (24)	8 (33.3)	16 (66.7)	7 (43.8)	9 (56.2)
PD* (8)	5 (62.5)	3 (37.5)	2 (66.7)	1 (33.3)

*Statistically significant difference of positive immunoreactivity for the 14-3-3σ between WD ECC and PD ECC by using Fisher's exact test (p= 0.031). Dx, diagnosis; IPNL, intraductal papillary neoplasms of the liver; ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

Table 3. Methylation status of the 14-3-3σ gene in biliary neoplasms

Pathologic Dx (Case No.)	14-3-3σ staining	Methylation status case No. (%)	
		Negative	Positive
IPNL (15)		8 (53.3)	7 (46.7)
	Negative	4 (50.0)	3 (42.9)
	Positive	4 (50.0)	4 (57.1)
Mass-forming ICC (14)		9 (64.3)	5 (35.7)
	Negative	3 (33.3)	5 (100.0)
	Positive	6 (66.7)	0 (0.0)
Non-papillary ECC (16)		10 (62.5)	6 (37.5)
	Negative	4 (40.0)	2 (33.3)
	Positive	6 (60.0)	4 (66.7)

Dx, diagnosis; IPNL, intraductal papillary neoplasms of the liver; ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma.
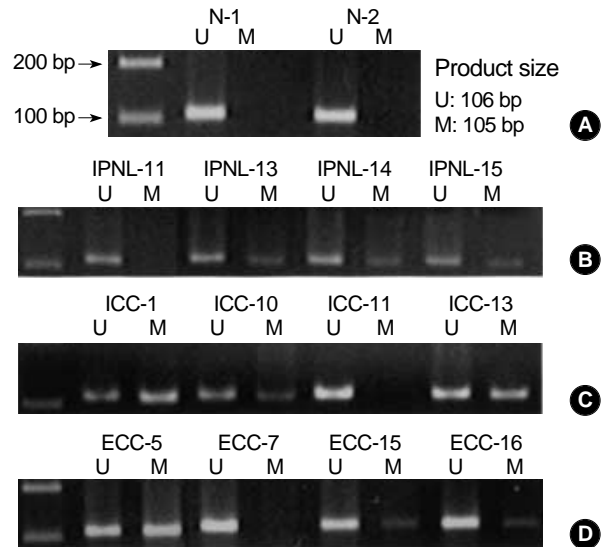

Fig. 2. Methylation status of the 14-3-3σ gene. (A) Non-neoplastic intrahepatic (N-1) and common bile ducts cells (N-2) show a 106 bp unmethylated band. Representative cases of IPNL (B), mass-forming ICC (C) and non-papillary ECC (D) show about 105 bp methylated bands (U, unmethylated; M, methylated).

the non-neoplastic intrahepatic and common bile ducts cells. Seven (46.7%) of 15 cases of IPNL (Fig. 2B), five (35.7%) of 14 cases of mass-forming ICC (Fig. 2C), and six (37.5%) of 16 cases of non-papillary ECC (Fig. 2D) showed hypermethylation of the 14-3-3σ gene promoter by MSP (Table 3). There was no significant difference in the methylation status of the 14-3-3σ gene among these 3 neoplastic groups (p=0.863). There was also no significant difference in the methylation status of the 14-3-3σ gene according to the types of IPNL and the differentiation of the mass-forming ICC and non-papillary ECC. Negative immunoreactivity for the 14-3-3σ protein was observed in three (42.9 %) of seven cases of IPNL with hypermethylation, in all five (100%) cases of mass-forming ICC with hypermethylation, and in two (33.3%) of six cases of non-papillary ECC with hypermethylation (Table 3).

DISCUSSION

In the present study, we first reported on the negative or weakly positive cytoplasmic immunoreactivity for the 14-3-3σ protein in the non-neoplastic intrahepatic and extrahepatic bile ducts cells. For the pancreatic ductal cells or acini, the negative[17,26] or weakly positive cytoplasmic[26] expression of the 14-3-3σ protein was reported, which was similar to that of the non-neoplastic bile duct cells in this study. However, a moderately to weakly positive cytoplasmic expression of the 14-3-3σ protein was reported in the gallbladder.[17]

We defined the positive immunoreactivity as well as the negative immunoreactivity that was associated with hypermethylation of the 14-3-3σ gene as the abnormal expression of the 14-3-3σ protein in biliary neoplasms on the basis of the findings in the non-neoplastic bile duct cells. We first demonstrated the abnormal expression of the 14-3-3σ protein in the majority of

the IPNLs (16/23, 69.6%), the mass-forming ICCs (21/33, 63.6%) and the non-papillary ECCs (27/38, 71.1%). The similar rates for the abnormal expression of the 14-3-3σ protein, regardless of the subtypes of biliary neoplasm, suggest its general association with biliary carcinogenesis.

In IPNL, the positive immunoreactivity for the 14-3-3σ protein showed a tendency to increase from the type 1 to type 4. Like the non-neoplastic intrahepatic bile duct cells, most cases of type 1 IPNL (3/4, 75.0%) revealed predominantly negative immunoreacitivity for the 14-3-3σ protein. The positive immunoreactivity for the 14-3-3σ protein in the majority of type 2, type 3 and type 4 IPNLs may suggest a compensatory increase of a negative regulator such as the 14-3-3σ protein against the overexpression of positive regulators during the progression of the cell cycle. In ICC, the overexpression of a positive regulator such as cyclin D1 was reported in the early cell cycle.[6] Furthermore, part of the newly expressed 14-3-3σ protein may show a membranous distribution, and this is due to an unknown mechanism. Whereas in type 5 IPNL, the increase of the negative immunoreactivity for the 14-3-3σ protein suggests its negative role for tumor invasion in the late stage of IPNL progression. As in biliary neoplasms, all the benign and borderline tumors, as well as the majority of malignant ovarian tumors, showed positive immunoreactivity for the 14-3-3σ protein; however, negative immunoreactivity for the 14-3-3σ protein was significantly correlated with a high tumor grade and an advanced tumor stage.[28] For the mass-forming ICC and non-papillary ECC (p= 0.031), the increased negative immunoreactivity for the 14-3-3σ protein in the PD tumor cells indicates a regulatory role of the 14-3-3σ protein during cellular differentiation. A similar result was reported in PD gastric adenocarcinomas in which the loss of the 14-3-3σ protein was much more frequently noted.[16] The positive immunoreactivity for the 14-3-3σ protein in the PD tumor cells of mass-forming ICC and non-papillary ECC may be associated with hypomethylation of the 14-3-3σ gene.

The 14-3-3σ protein has been reported to show cytoplasmic staining in most non-neoplastic tissues,[17] and the membranous accentuation of the 14-3-3σ protein has only been reported in pancreatic ductal adenocarcinomas in conjunction with cytoplasmic staining.[26] Thus, the membranous staining in the majority of biliary neoplasms in the current study appears to be a characteristic finding. For WD non-papillary ECC, the high frequency of the positive membranous expression of the 14-3-3σ protein (83.3%) may suggest its association with better cellular differentiation. However, further studies are needed to validate the membranous expression in IPNL and in the mass-forming ICC.

In the present study, hypermethylation was not detected in the non-neoplastic epithelium of both bile ducts. The methylation status of the 14-3-3σ gene in the non-neoplastic bile ducts has been described as unmethylated in the intrahepatic bile ducts,[5] and there has been no report on the methylation status of the 14-3-3σ gene in non-neoplastic extrahepatic bile duct cells. However, the frequency of the 14-3-3σ gene methylation in the mass-forming ICC (35.7%) was lower than that reported by Lee *et al*. (59.5%).[5] To regulate the expression of the 14-3-3σ protein, complex mechanisms appear to be involved in the different subtypes of biliary neoplasms. Although a complete loss of the 14-3-3σ protein was observed in all cases of mass-forming ICC with hypermethylation, more than half of the cases of IPNL (57.1%) and ECC (66.7%) maintained the expression of the 14-3-3σ protein despite the hypermethylation. These results may have come about from the heterogeneous mosaic methylation profile or due to the contamination of normal stromal cells. Some cases of IPNL (50%), the mass-forming ICC (33.3%) and the ECC (40%) showed negative immunoreactivity for the 14-3-3σ protein in the absence of methylation. The mechanism of these results is uncertain; however, incomplete induction due to the impairment of p53 function[12] or ubiquitin mediated proteasomal degradation[30] may be involved.

In conclusion, the similar rates of overexpression, as well as the loss of the 14-3-3σ protein in association with the 14-3-3σ gene hypermethylation, among the three groups of biliary neoplasms indicate its general association with biliary carcinogenesis. Furthermore, the increase of negative immunoreactivity in the cases of IPNL with definite invasion and in the PD ICC/ECC suggests that the 14-3-3σ protein may be involved in the progression of tumor and in the tumor cell differentiation of biliary neoplasms. However, the methylation status of the 14-3-3σ gene suggests a variable association with the down-regulation of the 14-3-3σ protein, according to the type of biliary neoplasm. The expression of the 14-3-3σ protein appears to be regulated by complex mechanisms, among the different subtypes of biliary neoplasms, and further studies are needed to clarify the mechanism.

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