

# Regulators of Apoptosis in Cholangiocarcinoma

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• **Context.**—Dysregulation of mediators of apoptosis is associated with carcinogenesis. For biliary duct cancers, *p53* gene mutation is an important contributor to carcinogenesis. Mutations in the *p53* gene affect transcription of the *Fas* gene, resulting in lack of Fas expression on cell membrane. It has been previously shown that cloned Fas-negative but not Fas-positive human cholangiocarcinoma cells are resistant to anti-Fas-mediated apoptosis and develop tumors in nude mice. In addition, interferon gamma induces Fas expression in Fas-negative cholangiocarcinoma cells and makes them susceptible to apoptosis. Therefore, it becomes important to characterize immunophenotypic expression of *p53* and Fas in normal and neoplastic human tissues of the biliary tract to further understand the pathogenesis of the disease. To date, human studies to characterize differences in immunophenotypic expression of the Fas protein between intrahepatic and extrahepatic biliary duct cancers and in their precursor lesions have not been performed.

**Objective.**—To report the immunophenotypic expression of *p53* and Fas expression in various stages in the development of bile duct cancers (intrahepatic and extrahepatic tumor location) and their association with tumor differentiation.

Carcinomas that arise from the biliary duct epithelium are rare. Intrahepatic bile duct cancers are commonly referred to as cholangiocarcinoma, whereas the extrahepatic tumor location is referred to as extrahepatic bile duct carcinoma. Intrahepatic cholangiocarcinoma demonstrates marked geographic variation in its incidence,<sup>1,2</sup> etiopathogenesis,<sup>1</sup> and genetic expression.<sup>2,3</sup> Such a variation is not observed with bile duct cancers that arise in the extrahepatic biliary tree.<sup>4</sup> Dysplasia is an intermediate step in the development of biliary tract cancer.<sup>5-7</sup> Understanding the underlying molecular phenotypic expression of biomarkers may provide an additional clue to the pathogenesis of bile duct cancers, predictive prognostic markers, and

**Design.**—Thirty bile duct cancer samples (13 intrahepatic and 17 extrahepatic) from 18 men and 12 women who ranged in age from 44 to 77 years (mean age, 65.6 years) were retrieved from the surgical pathology files. Hematoxylin-eosin-stained slides were evaluated for the type and grade of tumor and dysplastic changes in the biliary tract epithelium. Additional slides were immunohistochemically stained with *p53* and anti-Fas mouse monoclonal antibody. The pattern of Fas distribution and percentage of cells positive for *p53* and Fas expression were determined.

**Results.**—The percentage of Fas-expressing cells is significantly ( $P = .01$ ) more frequently noted in extrahepatic tumors compared with intrahepatic tumors. Furthermore, Fas expression decreased from dysplastic epithelium to cholangiocarcinoma ( $P = .01$ ), and this decreasing trend continued from well to poorly differentiated tumors. Nuclear *p53* expression was not identified in normal and dysplastic epithelium but was noted in 30% of carcinomas ( $P = .02$ ).

**Conclusion.**—Fas expression is an early event in pathogenesis of bile duct cancers. Immunophenotypic expression of Fas is associated with well to moderately differentiated tumors but not with poor tumor differentiation.

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molecular markers that may serve as potential therapeutic targets.

In many tumors, dysregulation of apoptosis plays a major role in pathogenesis of tumor development. Apoptosis is an active process initiated by either external or internal stimuli that results in a cascade of events that lead to eventual cell death.<sup>8-10</sup> Genetic and immunophenotypic abnormalities in *p53* in cholangiocarcinoma have been previously investigated.<sup>11-17</sup> These results have provided mixed results. Mixed results in the literature for association of *p53* in the development of biliary duct carcinomas may be a reflection of many factors, including the location of samples obtained from the biliary tree, sample processing, and underlying genetic variation in geographic regions.<sup>3,11,14,15</sup> It is also noted that mutation in the *p53* gene may affect transcription of the *Fas* gene.<sup>18</sup>

In recent years, the role of the Fas/Fas ligand (Fas/FasL) apoptotic signaling pathway in carcinogenesis has been increasingly investigated.<sup>10,19-23</sup> Fas (CD95/APO-1) is a cell surface membrane receptor of the tumor necrosis factor superfamily present on many epithelial cells. Binding of FasL to Fas trimerizes the intracellular domain of Fas (death domain) that attaches to adapter intracellular proteins (FADD/MORT1) and activates caspases that execute the process of apoptosis.<sup>8,9</sup> Expression of Fas on the cell

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surface is necessary to initiate apoptosis. Its absence, however, may allow continuous proliferation of genetically unstable cells that may undergo malignant transformation.

Only a few studies have examined the role of Fas in cholangiocarcinogenesis.<sup>22,24-28</sup> Our *in vitro* experiments have demonstrated that only cloned Fas-positive human cholangiocarcinoma cells are susceptible to anti-Fas-mediated apoptosis.<sup>22</sup> It is also noted that Fas-negative but not Fas-positive cloned human cholangiocarcinoma cells induce tumor development in nude mice.<sup>22</sup> Furthermore, it is also noted that tamoxifen, a known anticancer agent, and interferon gamma can induce tumor regression in xenograft models developed from cloned Fas-negative human cholangiocarcinoma cells.<sup>25,26</sup> Clearly, these studies show that Fas expression is a key determinant of cholangiocarcinogenesis. These studies also make a compelling case that Fas expression in cholangiocarcinoma can serve as a potential molecular target for treatment of cholangiocarcinoma. The aforementioned studies therefore suggest that the next important logical step would be to undertake translational studies to examine molecular events associated with various stages in the development of human bile duct carcinomas.

The objective of the present study was to characterize immunophenotypic expression of p53 and Fas in the normal biliary epithelium, biliary tract dysplasia, and bile duct carcinoma (intrahepatic and extrahepatic). Furthermore, this study was undertaken to determine the pattern and association of Fas expression with tumor differentiation in intrahepatic cholangiocarcinoma and extrahepatic bile duct carcinomas.

## MATERIALS AND METHODS

Surgical pathology samples from 30 bile duct cancers (13 intrahepatic and 17 extrahepatic) from 18 men and 12 women who ranged in age from 44 to 77 years (mean age, 65.6 years) were obtained from the surgical pathology files of 2 major tertiary care institutions. Formalin-fixed, paraffin-embedded, hematoxylin-eosin-stained sections were reviewed, and histologic type and tumor grade were further characterized based on their growth pattern and degree of cellular anaplasia based on the recently published criteria.<sup>24</sup> The tumor staging was also determined at the time of surgical resection based on the AJCC *Cancer Staging Manual*.<sup>29</sup> In addition, adjacent normal and dysplastic tissues were also examined histologically from the same patient sample.

The sections were then immunohistochemically stained with mouse monoclonal antibody against Fas (CD95/APO-1) using the following protocol. Tissue sections were deparaffinized in xylene, rehydrated through decreasing concentrations of alcohol ending in phosphate-buffered saline (PBS), and placed in proteinase K. The sections were quenched with 3% hydrogen peroxidase, incubated with protein block (Dako Corporation, Carpinteria, Calif) for 15 minutes at room temperature, and washed in PBS. Tissues were then incubated with mouse anti-Fas antibody (clone, APO-1; dilution, 1:10; incubation, 80 minutes at room temperature; Dako). Finally, sections were washed in PBS with 0.05% Tween 20 (pH 7.4), and the bound antibody was detected using streptavidin and biotinylated secondary antibody reacted with diaminobenzidine as chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were sections treated as described herein, but instead of incubation with the primary antibody they were incubated with 1% bovine serum albumin and PBS. The pattern of immunoreactivity for Fas was noted and was considered positive when membrane staining was identified. For purposes of evaluation, staining in greater than 10% of cells was considered positive.

Immunohistochemical analysis for p53 was performed using

the following protocol. Tissue sections were deparaffinized in xylene and rehydrated through decreasing concentrations of alcohol, ending in PBS. Sections were then incubated with the anti-p53 monoclonal antibody BP53 (clone, 12-1; dilution, 1:40; BioGenex, San Ramon, Calif) for 1 hour at room temperature, and preimmune rabbit serum was used to test for nonspecific staining and reactivity of the secondary detection system with the tissue. The bound primary antibody was detected using streptavidin and biotinylated secondary antibody using the BioGenex super-sensitive detection kit and then reacted with diaminobenzidine as chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted. Only those tumor cells with distinct nuclear immunostaining for p53 in at least 10% of cells were considered as stain positive.

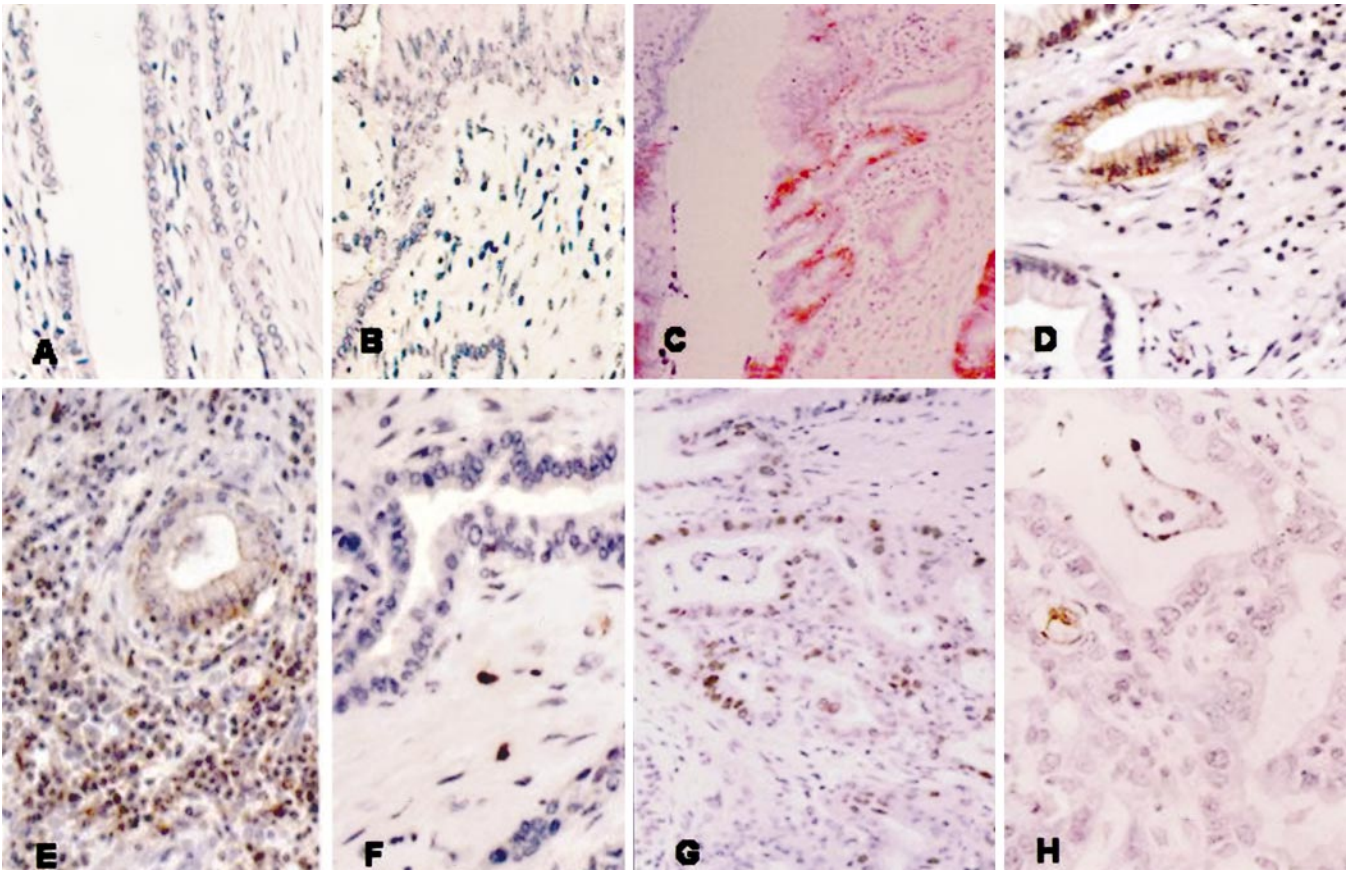
Wilcoxon 2-sample tests and the  $\chi^2$  test were performed to evaluate statistical associations using the SAS statistical software (SAS Institute Inc, Cary, NC). All tests of significance were 2-sided with  $\alpha = .05$ .

## RESULTS

Of the 30 patient samples, adjacent normal epithelium from large caliber bile ducts were noted in 12, dysplasia in 11, and carcinoma in 30. Of the 30 patients with invasive carcinoma, 23 were characterized as having well to moderate differentiation and 7 had poor differentiation. Four of these 30 samples were invasive papillary carcinoma, and 26 adenocarcinoma were not otherwise specified.

None of the samples demonstrated p53 nuclear expression in normal (Figure, A) and dysplastic (Figure, B) biliary tract epithelium. In comparison, p53 nuclear protein expression was significantly more frequently (9/30,  $P = .02$ ) expressed in bile duct carcinomas and when present was noted in a mean of 10% of tumor cells. Fas protein expression was not detected in most samples (7/12, 58%) from adjacent normal-appearing biliary tract epithelium (Figure, C). When present, it was weakly expressed on average in 10% of cells. In comparison, all 11 samples (100%) with dysplasia expressed Fas protein in dysplastic epithelium (Table 1). In addition, mean Fas expression was significantly ( $P = .01$ ) increased in dysplastic epithelium compared with the normal epithelium (Table 1 and Figure, C). In bile duct carcinomas, a fewer number of samples (17/30, 57%) detected Fas expression compared with the dysplastic epithelium (11/11, 100%) (Table 1). Carcinoma cells also demonstrated a mean of 28% of cells that expressed Fas protein, which is significantly increased from adjacent normal epithelium ( $P = .01$ ). These observations show a trend where number of samples and mean Fas expression increased in dysplastic epithelium compared with the normal and then was again lost from carcinoma cells.

In carcinoma cells, Fas expression was preferentially noted on the cell membrane (Figure, D). In addition, marked lymphocytic response, a measure of cell-mediated immune reaction, was noted around Fas-positive (Figure, E) but not Fas-negative tumor cells (Figure, F). Increased lymphocytic response was noted around 60% of Fas-positive tumor cells. In comparison, only 12% of Fas-negative tumor cells demonstrated increased lymphocytic response. Most samples with well or moderately differentiated tumors (15/23, 65%) demonstrated Fas expression compared with poorly differentiated (2/7, 29%) tumors (Table 1). Mean p53 and Fas expression was significantly ( $P = .01$ ) decreased in poorly differentiated tumors compared with well and moderately differentiated tumors (Table 2). Fas



A, Tissue section shows no p53 immunoreactivity in normal biliary tract epithelium (immunohistochemistry for p53, original magnification  $\times 40$ ). B, Tissue section shows no immunoreactivity for p53 in dysplastic biliary tract epithelium (immunohistochemistry for p53, original magnification  $\times 40$ ). C, Tissue section shows no immunoreactivity in the adjacent normal biliary tract epithelium but shows immunoreactivity in the dysplastic epithelium (immunohistochemistry for Fas [CD95/APO-1], original magnification  $\times 4$ ). D, Extrahepatic bile duct carcinoma with immunohistochemical expression of Fas (CD95/APO-1) on cell surface membrane (immunohistochemistry for Fas [CD95/APO-1], original magnification  $\times 40$ ). E, Extrahepatic bile duct carcinoma with increased Fas-positive lymphocytes noted around Fas-positive tumor cells (immunohistochemistry for Fas [CD95/APO-1], original magnification  $\times 20$ ). F, Extrahepatic bile duct carcinoma with no lymphocytes around Fas-negative tumor cells (immunohistochemistry for Fas [CD95/APO-1], original magnification  $\times 20$ ). G, Bile duct carcinoma with nuclear p53 protein expression (immunohistochemistry for p53, original magnification  $\times 20$ ). H, Serial section from same tumor with area as shown in G shows lack of Fas expression (immunohistochemistry for Fas [CD95/APO-1], original magnification  $\times 40$ ).

**Table 1. p53 and Fas Expression and Tumor Characteristics**

	No. of p53-Positive Samples	P Value	No. of Fas-Positive Samples	P Value
<b>Tumor differentiation</b>				
Adjacent normal (n = 12)	0		5	
Dysplasia (n = 11)	1		11	.01
Carcinoma (n = 30)	9	.02	17	.01
<b>Tumor location</b>				
Intrahepatic (n = 13)	3		4	
Extrahepatic (n = 17)	6	.06	13	.02
<b>Tumor grade</b>				
Well to moderate (n = 23)	9	.05	15	.02
Poor (n = 7)	0		1	

expression was only focally noted in all tumors categorized as invasive papillary carcinoma. Poorly differentiated areas in the tumor with predominantly well to moderate differentiation also demonstrated none or weak staining. In serial sections, tumor cells that demonstrated

nuclear staining for p53 (Figure, G) usually did not show Fas expression (Figure, H).

We also noted tumor location-associated differences in Fas protein expression. Thirteen (76%) of 17 extrahepatic bile duct carcinomas demonstrated Fas expression com-

	Mean p53 Expression, %	Mean Fas Expression, %	P Value
Tumor differentiation			
Adjacent normal (n = 12)	0	10	
Dysplasia (n = 11)	0	39	.01
Carcinoma (n = 30)	10	28	.01
Tumor location			
Intrahepatic (n = 13)	10	10	.02
Extrahepatic (n = 17)	30	20	
Tumor grade			
Well to moderate (n = 23)	20	18	.01
Poor (n = 7)	0	6	

Stage	Fas Expression		
	0%–10%	11%–50%	>50%
Extrahepatic			
Stage I (n = 2)		1	1
Stage II (n = 2)	1	1	
Stage III (n = 0)			
Stage IV (n = 13)	3	6	4
<b>Total (n = 17)</b>	<b>4</b>	<b>8</b>	<b>5</b>
Intrahepatic			
Stage I (n = 0)			
Stage II (n = 0)			
Stage III (n = 12)	8	2	2
Stage I (n = 1)	1		
<b>Total (n = 13)</b>	<b>9</b>	<b>2</b>	<b>2</b>

pared with only 4 (31%) of 13 intrahepatic tumors (Table 1). Furthermore, a significant difference ( $P = .02$ ) in mean Fas expression was noted between intrahepatic and extrahepatic tumors (Table 2).

Twenty-six (87%) of the 30 samples with bile duct carcinoma were in the advanced stage (stages III and IV) (Table 3). In these tumors, statistical analysis for stage-matched carcinomas at different tumor locations (intrahepatic and extrahepatic carcinomas) and Fas protein expression was not performed due to smaller sample size, which could not provide enough statistical power. Interestingly, Fas expression was noted with a higher frequency of 77% (10/13) in patients with advanced stage (stages III to IV) extrahepatic tumors compared with only 31% (4/13) in advanced stage intrahepatic carcinomas.

#### COMMENT

Prognosis for extrahepatic bile duct carcinomas remains poor regardless of the histologic grade or stage.<sup>4</sup> The results from the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute during a 10-year period (1977–1986) show that the 2-year relative survival rate for the extrahepatic biliary duct carci-

noma patients with histologic grades 1 and 4 are 28% (median survival, 10 months) and 0% (median survival, 2 months), respectively.<sup>30</sup> The SEER data also demonstrated that the 2-year relative survival for stages I, III, and IV for extrahepatic biliary duct carcinoma is 27% (median survival, 12 months), 17% (median survival, 8 months), and 4% (median survival, 3 months), respectively.<sup>30</sup> Recently, it was noted that the median survival for resected intrahepatic, perihilar, and distal tumors is 26, 19, and 22 months, respectively.<sup>31</sup> Multivariate analysis has demonstrated that after curative resection, lymph node metastasis and less differentiated histologic type were significant risk factors for poor outcome in patients with intrahepatic cholangiocarcinoma.<sup>32</sup> In this study, all 6 of the 37 patients who survived more than 5 years had undergone curative resection for well-differentiated adenocarcinoma.<sup>32</sup> These studies demonstrate that both stage and tumor differentiation play a significant role in patient survival following surgical resection.

Underlying molecular phenotypic expression of biomarkers may provide an additional clue to the development of cholangiocarcinoma and predictive prognostic molecular markers that also may serve as potential therapeutic targets. Relatively few studies have directed their efforts to understanding the role of mediators of apoptosis, their immunohistochemical associations, importance in the development of cholangiocarcinoma, and their potential therapeutic implications. Investigations into the role of apoptosis and its mediators in cholangiocarcinoma by Terada and Nakanuma<sup>13</sup> have demonstrated that 40% of human cholangiocarcinoma tissues expressed p53 but none expressed Fas and c-Myc. In contrast to the study by Terada and Nakanuma in which Fas was not observed in cholangiocarcinoma tissues, we have now conclusively demonstrated that Fas expression can be observed in human biliary duct cancers (both in intrahepatic and extrahepatic locations). This expression is observed on the cell surface membrane and is in keeping with both in vitro<sup>22,25,26</sup> and other human tissue studies.<sup>33,34</sup>

The data in the present study are consistent with up-regulation of Fas being an early change in the development of cholangiocarcinoma. Our study shows that 100% of the samples with dysplastic epithelium, 65% with well to moderately differentiated carcinoma, and 29% of poorly differentiated biliary duct carcinomas expressed Fas. Another molecular marker, p53, has been investigated relatively more extensively and has provided mixed results.<sup>13–16,35,36</sup> Similar to our observations, Arora et al,<sup>16</sup> who studied 28 cases of cholangiocarcinoma, concluded that p53 expression is a late event in the development of cholangiocarcinoma. They show that most adenocarcinoma expressed p53, whereas none of the adjacent bile duct or dysplastic epithelium demonstrated its expression.<sup>16</sup> Similar to our observation, Caca et al<sup>37</sup> also found 7 (33%) of 21 tumors positive for p53 by immunohistochemical staining. In their study, 50% of grade 3 tumors did not show p53 by immunohistochemical staining. How underlying genetic abnormalities may be associated with immunohistochemical expression of p53 protein is a subject of continued investigations. It has been suggested that mutations in the *p53* gene may result in a stable protein product that may be accumulated in the nucleus and detected on immunohistochemical stains. Mutations in the *p53* gene have been noted in bile duct and gall bladder carcinomas.<sup>38,39</sup> In their study of 12 Klatskin tumors, Jonas et al<sup>39</sup> dem-

onstrated mutational hotspots in the *p53* gene in 4 patients. These investigators, however, could not detect *p53* nuclear protein by immunohistochemical analysis in any of the tumors with mutational hotspots. Interestingly, the study by Caca et al<sup>37</sup> shows *p53* immunohistochemical reactivity in 32% of the patients but did not demonstrate any mutations in exons 5 to 8 of *p53* either by single-stranded conformation polymorphism analysis or direct sequencing. Caca et al also did not detect mutations in any of the 3p16 exons, which may affect transcription of the *p53* gene. However, these investigators detected hypermethylation in the promoter region of the *p16* gene in 43% of their tumor samples and correlated this with lack of *p16* immunohistochemical expression. Their results suggest that altered *p53* expression in a subset of patients with bile duct cancers could be a result of genetic alteration in *p53* regulatory genes.

It was recently reported that moderate (40%–50%) down-regulation of CD95 messenger RNA and surface protein expression occurs in the presence of mutant *p53*, which correlates with partial protection against CD95-dependent cell death. In addition, excess *p53* mutation has demonstrated repression of the CD95 promoter activity, which affects transcription of the *Fas* gene. The latter study supports our observation that *Fas* expression is an early event in the development of cholangiocarcinoma and a subsequent mutation in the *p53* gene will result in down-regulation and loss of *Fas* gene transcription, resulting in loss of its expression in biliary duct carcinomas.

Up-regulation of *Fas* at early stages in the development of bile duct carcinomas may also represent a host response to help the cell-mediated immune system eliminate genetically unstable cells via *Fas*/*FasL*-mediated apoptosis. Expression of *Fas* on the cell surface membrane predisposes a cell to immune elimination by interaction with *FasL* produced by the host's T lymphocytes recruited to the vicinity.<sup>19</sup> In the present study, we also observed that lymphocytes, the mediators of the cell-mediated immune response, are noted more frequently around *Fas*-positive but not *Fas*-negative cells. In a recent study,<sup>40</sup> *FasL* expression was noted in 12 of 15 reactive lymph nodes. It has also been repeatedly demonstrated that *FasL* is detected in tumor-associated lymphocytes in cholangiocarcinoma tissues using either messenger RNA detection or immunohistochemical expression using 2 commercially available antibodies to *FasL*.<sup>33,34</sup> It has also been documented that *FasL* expression is noted in at least one third of lymphoid cells that infiltrate cholangiocarcinoma cells and those remote from the tumor cells.<sup>34</sup>

In the present study, *Fas* expression in both intrahepatic and extrahepatic bile duct cancers was determined. Our study demonstrates that *Fas* protein expression was noted with lower frequency in intrahepatic cholangiocarcinoma (8/13, 62%) compared with extrahepatic bile duct carcinoma (15/17, 88%) and that the difference in the mean *Fas* expression in tumor cells was statistically significant ( $P = .02$ ). In comparison, Shimonishi et al,<sup>34</sup> who examined *Fas* expression in only intrahepatic cholangiocarcinoma, noted its immunohistochemical expression in a higher percentage of cases (55/68, 81%) compared with the present study (8/13, 62%). The differences in *Fas* expression based on the anatomic location and in same location between 2 geographically diverse areas may suggest inherent genetic and environmental factors, which may affect molecular pathways in the development of cho-

langiocarcinoma. In a study of 128 bile duct carcinomas (intrahepatic, proximal, and distal locations), Argani et al<sup>14</sup> showed differences in immunophenotypic expression of *p53* in different anatomic locations with increased *p53* expression in distal common bile duct tumors compared with the proximal location (51% vs 26%,  $P < .001$ ). These studies show that even in the biliary tract epithelium there are differences in the molecular expression patterns at different locations, which may result in activation or deactivation of different tumor-promoting activities and finally affect tumor progression.

This study shows that *Fas* expression is significantly ( $P = .01$ ) decreased in poorly differentiated tumors compared with well to moderately differentiated tumors in both intrahepatic and extrahepatic tumors. Similar to our observation, Shimonishi et al,<sup>34</sup> who used the same antibody clone (clone, APO-1; Dako) as our study, showed that a higher percentage of tumors with poor differentiation (9/27, 33%) lack *Fas* protein expression compared with either well (1/22, 5%) or moderately (3/19; 16%) differentiated tumors. Moreover, most of our intrahepatic tumors are higher stage and show decreased *Fas* expression. This down-regulation of *Fas* in poorly differentiated tumor cells is most likely associated with resistance to *Fas*/*FasL*-mediated apoptosis.

It has been previously demonstrated that *Fas* expression offers a survival advantage to patients with cholangiocarcinoma.<sup>34</sup> In vitro studies in our laboratory have demonstrated that *Fas*-negative but not *Fas*-positive cloned human cholangiocarcinoma cells (SK-CHA-1) are resistant to apoptosis and produce tumors in nude mice.<sup>22</sup> We have also demonstrated marked up-regulation of *Fas* and downstream apoptosis-effector proteins in *Fas*-negative human cholangiocarcinoma cells following treatment with interferon gamma. These cells show increased susceptibility to anti-*Fas*-mediated apoptosis<sup>26</sup> and reduce tumor volume in xenografts induced in nude mice.<sup>25</sup> In these studies, *Fas* expression is correlated with the ability of a class of  $CA^{2+}$  signaling inhibitors (calmodulin antagonists) to stimulate apoptosis.<sup>26</sup> Two potent calmodulin antagonists, tamoxifen and trifluoperazine (Stelazine), are well tolerated by patients. Both tamoxifen and trifluoperazine can induce apoptosis only in *Fas*-positive cholangiocarcinoma cells<sup>22</sup> and decrease tumor cell growth in vitro.<sup>22</sup> It is also possible that *Fas* expression in cholangiocarcinoma could be used as a biomarker for a therapeutic decision for these highly malignant tumors.

In summary, *Fas* expression is not only present on cholangiocarcinoma cells but is also an early event in the development of cholangiocarcinoma and is associated with tumor differentiation. There are differences in the frequency of *Fas* expression between intrahepatic and extrahepatic bile duct cancers, which may affect tumor development by activation or deactivation of different tumor-promoting activities.

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