INTERNAL MEDICINE

Slowly Progressive Insulin-Dependent Diabetes in a Patient with Primary Biliary Cirrhosis with Portal Hypertension-Type Progression

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Abstract

A 73-year-old woman had previously been diagnosed with CREST syndrome, PBC and diabetes. Hepatic fibrosis was not evident, in spite of the transudative ascites and active esophageal varices. ACA were positive, whereas AMA and anti-gp210 antibodies were negative. She showed low urinary excretion of C-peptide and was weakly positive for anti-GAD antibody. She was diagnosed with a form of PBC that progresses via portal hypertension rather than liver failure and with SPIDDM. Her HLA type did not contain risk allele for IDDM or PBC. SPIDDM should be considered when patients with PBC with portal hypertension-type progression develop diabetes.

Key words: SPIDDM, primary biliary cirrhosis, portal hypertension, anti-centromere antibodies (ACA), CREST syndrome

Case Report

A 73-year-old woman was admitted to our hospital in April 2009 for control of diabetes and ascites. She had no family history of diabetes or liver disease. She had developed Raynaud's phenomenon at 40 years of age and sclerodactyly when she was 44. She had been diagnosed with calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST) syndrome based on her clinical features and the presence of anti-centromere antibodies (ACA). At 65 years of age, a routine screening revealed abnormal serum alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GTP) levels, postprandial hyperglycemia, and elevated levels of hemoglobin A1c (HbA1c). She did not have a history of alcohol or drug abuse, and was negative for anti-mitochondrial antibodies (AMA), the

M2 fraction of AMA, and viral markers for hepatitis B and C. A liver biopsy showed chronic non-suppurative destructive cholangitis (CNSDC)-like bile duct injuries with granulomatous reactions, and intraepithelial lymphocytic infiltration (Fig. 1A). Based on these findings, the patient was diagnosed with primary biliary cirrhosis (PBC) and diabetes. After ursodeoxycholic acid (UDCA) and sulfonylurea (glimepiride 1 mg) were administered, her ALP level remained high, but her diabetes was well controlled.

In June 2009, at 73 years of age, the patient was referred to our hospital because of ascites and worsening diabetes. She presented with anemia, skin thickening, sclerodactyly, palmar erythema without vascular spider, hepatomegaly, an abdominal fluctuation suggestive of ascites, and edema of the leg. Her laboratory data (Table 1) showed elevations in ductal enzyme levels and a preserved hepatic reserve. Her diabetes was poorly controlled (FPG, 341 mg/dL; HbA1c,
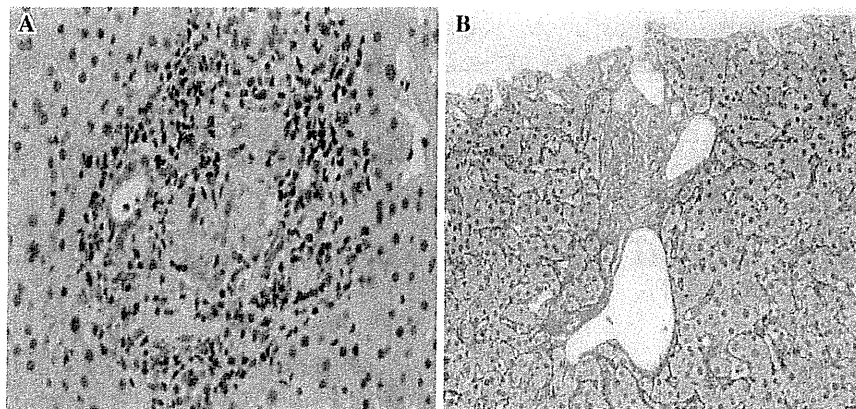
Figure 1.   Histological findings from liver needle biopsy specimens. A. Damaged interlobular bile duct associated with a granulomatous reaction. B. The portal tract is sclerotic, and an abnormal blood vessel herniation in the parenchyma is present.

Table 1.   Laboratory Data on Admission

WBC	6320	/μL	BUN	28	mg/dL	IgG	893	mg/dL
RBC	2.86×10^6	/μL	Cr	0.39	mg/dL	IgA	389	mg/dL
Hb	9.9	g/dL	ALP	1725	IU/L	IgM	726	mg/dL
Ht	23.3	%	GTP	142	IU/L	Anti-TPO Ab	2.7	IU/mL
Plts	261×10^3	/μL	AST	19	IUL	Anti-Tg Ab	2880	IU/mL
FPG	341	mg/dL	ALT	29	IU/L	HBsAg	Negative	
HbA1c	9.1	%	Amy	28	IU/L	HCVAb	Negative	
(JDS value)			T-Bil	0.4	mg/dL	ANA	640	
			TP	5.7	g/dL	AMA	Negative	
			Alb	3.9	g/dL	AMA-M2	Negative	
			PT	92	%	Anti-GAD Ab	3.2	IU/mL
			CPR	0.3	ng/mL	IA-2 Ab	<0.4	IU/mL
						ACA	120.2	
						Anti-gp210 Ab	0.7	

JDS, Japanese Diabetes Society; PT, prothrombin time; Anti-TPO Ab, thyroperoxidase antibody; Anti-Tg Ab, thyroglobulin antibody; HBsAg, hepatitis B virus surface antigen; HCV, antibody to hepatitis C virus; ANA, anti-nuclear antibodies; AMA, anti-mitocondrial autoantibodies ; Anti-GAD Ab, glutamic acid decarboxylase antibody; IA-2 Ab, insulinoma-associated antigen-2; ACA, anti-centromere antibodies

9.1%). She was positive for various autoantibodies, including anti-glutamic acid decarboxylase antibody (GAD Ab), Tg antibodies, and anti-centromere antibodies, but she was negative for AMA. Her human leukocyte antigen (HLA) type was DRB1*010101, DQB1*050101, DPB1*020102/0501, DQA1*0101.

Abdominal CT revealed hepatomegaly and collateral vascularization, in the paraesophageal region. Gastrointestinal endoscopic examinations showed esophageal varices (linear and white varices without red coloring). We performed hepatic venography to further investigate the collateral vascularization and esophageal varices. The patient's wedged hepatic vein pressure (WHVP) was 11 mmHg and her hepatic venous pressure was 4 mmHg. The normal Hepatic Venous Pressure Gradient (the difference between the WHVP and the free hepatic venous pressures) value is between 1 and 5 mmHg (1). However, while transudative ascites were present, there was no evidence of portal hypertension. A needle liver biopsy was performed. The lobular architecture was relatively normal, and there was dense portal fibrosis without bridging. At the edges of the portal areas, abnormal blood vessels, frequently reported in idiopathic portal hypertension (2), were observed (Fig. 1B). Cholangitis, which had been detected in a previous biopsy (Fig. 1A), was not found.

Concerning the etiology of diabetes, the GAD Ab titer was 3.2 U/mL (normal range [NR], <1.5 U/mL). Plasma levels of basal circulating C-peptide immunoreactivity (CPR) and urinary excretion of CPR were as low as 0.3 ng/mL (NR, 0.94-2.8 ng/mL) and 12 μg/day (NR, 20.5-198 μg/day), respectively. We examined the responses of CPR and glucagon to arginine in the arginine challenge test. ΔCPR and Δglucagon were calculated from the difference between peak values and the base values of CPR and glucagon. Arginine challenge yielded a weak CPR response (ΔCPR, 0.1 ng/mL) and an exaggerated glucagon response (Δglucagon, 382 pg/mL), which are characteristic of type 1 diabetes (3). Based on these findings, a diagnosis of slowly progressive insulin-dependent diabetes mellitus (SPIDDM) was made. Basal-bolus insulin therapy (28 U/day of insulin lispro and 6 U/day of insulin glargine) reduced the patient's HbA1c to

80

6.5% after 6 months.

Discussion

Collectively, the present patient was diagnosed as having PBC, type 1 diabetes and autoimmune thyroid disease (AITD) based on positivity in Tg antibody and a heterogeneous internal echo finding of the thyroid. Therefore, it may be possible that our patient is included in the entity of autoimmune polyglandular syndrome type 3 that is composed of type 1 diabetes and AITD (4). In gerenal, the prevalence of GAD Ab is significantly higher in patients with AITD than in healthy control subjects (5). However, the present patient's HLA type was different from the frequent DRB1*0405-DQB1*0401 haplotype observed in type 1 diabetes and AITD (5). Chronic liver disease has also been implicated as a complication of Hashimoto's thyroiditis, and the term "hepatothyroiditis syndrome" has been proposed to describe this condition (6). Thyroid disease is also found in about 10-15% of patients with PBC, and AITD is the most common (7). The prevalence of GAD Ab is 5.5% in patients with PBC, higher than that in the healthy population (8). However, to the best of our knowledge, this is only the second reported case of SPIDDM complicated by PBC. SPIDDM (9), which is also referred to as latent autoimmune diabetes in adults (10), generally occurs in adults after a clinical course involving the control of type 2 diabetes with oral hypoglycemic agents. Because the level of GAD Ab was relatively low in the present patient, we should rule out insulinopenic type 2 diabetes in our patient. In this regard, we previously reported that arginine-induced CPR and glucagon responses were negatively and positively correlated with each other in patients with type 1 and type 2 diabetes, respectively (3). Autoimmune type 1 diabetes is caused by a targeted immune reaction that destroys β-cells while leaving the α-cell mass relatively unaffected (11). Therefore, intra-islet insulin deficiency determines the exaggerated glucagon response to arginine in type 1 diabetes. Because the present patient showed a diminished insulin response and exaggerated glucagon response to arginine challenge, we came to the conclusion that our patient has type 1 diabetes rather than type-2 diabetes.

There are at least two different types of progression in PBC: hepatic failure-type progression, which is characterized by the presence of anti-gp210 antibodies, and portal hypertension-type progression, which is characterized by the presence of ACA (12). The present patient was positive for ACA and was therefore deemed to be at high risk of portal hypertension-type progression, rather than hepatic failure-type progression (12). Indeed, she presented with portal hypertension with transudate ascites and esophageal varices without advanced liver fibrosis. The reasons for the different types of progression are not known, but one could argue that specific immunological interactions in the presence of an additional autoimmune disorder may influence the clinical picture and favor a better liver disease outcome (13).

It might be possible that the pathology of SPIDDM and CREST-PBC overlap syndrome are associated in the present patient because exacerbation of diabetes and portal hypertension developed in parallel in the clinical course. SPIDDM and PBC are both autoimmune diseases and share common features. For example, infiltration of CD8+T lymphocytes occurs in the exocrine pancreas in SPIDDM (14) and peripheral damage to the bile ducts is seen in PBC (15). Thus, CD8+T lymphocytes may have played a pathological role in the present case. On the other hand, the HLA susceptibilities in type 1 diabetes and CREST-PBC overlap syndrome are different. HLA-DQA1*0301-DQB 1*0401 haplotype is often present in SPIDDM (16), whereas HLA-Cw6 is often present and HLA-DR2 is often absent in CREST-PBC overlap syndrome (17). The HLA type of the present patient was different than other reported cases of IDDM and PBC. Thus, accumulation of the similar cases complicated with SPIDDM and CREST-PBC overlap syndrome will be necessary to shed light on the common pathology and genetic basis of this condition.

Generally, patients with PBC and collagen disease have lower rates of liver transplantation and liver-related death, and a slower rate of increase in bilirubin levels, compared to patients with PBC alone (18). More attention should be paid to the progression of portal hypertension (i.e., varices and ascites) and diabetic complications, which might be determinants of prognosis.

As a lesson from this case, we suggest that SPIDDM should be considered when patients with CREST-PBC overlap syndrome with portal hypertension-type progression develop diabetes.

The authors state that they have no Conflict of Interest (COI).

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Beyond "Cirrhosis"

A Proposal From the International Liver Pathology Study Group

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Upon completion of this activity you will be able to:
- define advanced-stage chronic liver diseases as dynamic conditions, characterized by remodeling of the hepatic pathologic changes.
- discuss the relevance of various approaches in assessing advanced chronic liver diseases, such as liver biopsy, hepatic venous pressure gradient, and transient elastography.
- compare the benefits of the contemporary clinicopathologic approach vs the traditional approach of assessment of the presence or absence of cirrhosis, in managing patients with advanced-stage chronic liver disease.

Abstract

"Cirrhosis" is a morphologic term that has been used for almost 200 years to denote the end stage of a variety of chronic liver diseases. The term implies a condition with adverse prognosis due to the well-known complications of portal hypertension, hepatocellular carcinoma, and liver failure. However, recent advances in the diagnosis and treatment of chronic liver diseases have changed the natural history of cirrhosis significantly. This consensus document by the International Liver Pathology Study Group challenges the usefulness of the word cirrhosis in modern medicine and suggests that this is an appropriate time to consider discontinuing the use of this term. The role of pathologists should evolve to the diagnosis of advanced stage of chronic liver disease, with emphasis on etiology, grade of activity, features suggestive of progression or regression, presence of other diseases, and risk factors for malignancy, within the perspective of an integrated clinicopathologic assessment.

Definition and Current Understanding of Cirrhosis

"Cirrhosis" derives from the Greek word κίρρος, meaning tawny, and was initially used to describe the gross (tawny, nodular, and firm) and, afterwards, the microscopic appearance of the chronically diseased and physiologically burned out and dysfunctional liver.[1,2] For almost 2 centuries, the emphasis was placed on the irreversible, "end-stage" nature of these livers; etiology was not considered very important because there was no cure, and survival was usually short. In 1977, an international panel, sponsored by the World Health Organization, defined cirrhosis as "a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules."[3] The panel also noted that "cirrhosis is a chronic progressive condition that results in liver cell failure and portal hypertension." Furthermore, the panel stated that vascular abnormalities were a very important feature of cirrhosis. The latter include thrombosis, obliteration, and recanalization of veins; formation of arteriovenous shunts; and "capillarization" of sinusoids.[4]

The natural history of cirrhosis has changed significantly in recent years, as therapeutic advances in the field of chronic liver diseases allow patients with cirrhosis to survive long-term, often with their conditions improving clinically and histologically, in the course of time. Therefore, it is becoming obvious that, by modern standards, all cases of cirrhosis do not inevitably "result in liver cell failure and portal hypertension." Furthermore, the possibility of regression of cirrhosis has been considered[5] and is now thought likely by several

investigators. From a practical viewpoint, a need to redefine cirrhosis as a pathologic condition that evolves through more than one stage has been acknowledged.[6]

Using a morphology-based unitary term, such as cirrhosis, for a part of the evolutionary spectrum of a variety of diseases is unusual in modern medicine because disease classification, diagnosis, and treatment are primarily based on etiology. Indeed, it is difficult to find any term referring to a pathologic condition of other organs that is conceptually similar to cirrhosis of the liver. Furthermore, the concept of cirrhosis as an end-stage and irreversible process is so widely known to the public that the implication of the word itself is often problematic.

Recent discussions by the International Liver Pathology Study Group (San Francisco, CA, 2009, and London, England, 2010) have suggested that this is an appropriate time to consider discontinuing use of the word cirrhosis. The role of pathologists should evolve to the diagnosis of advanced stage of chronic liver disease, with emphasis on etiology, grade of activity, features suggestive of progression or regression, presence of other diseases, and risk factors for malignancy, within the perspective of an integrated clinicopathologic assessment. The current article elaborates on this suggestion.

Cirrhosis and Cirrhoses

Cirrhosis is a heterogeneous condition with differing clinical manifestations and prognosis depending on the etiology and the severity of hepatic architectural distortion. The main causes of cirrhosis include chronic hepatitis B, chronic hepatitis C, autoimmune hepatitis, fatty liver diseases, chronic biliary diseases, and several inherited metabolic disorders.[4,7] Each one of these diseases has a relatively well-understood natural history and may cause significant liver injury, accompanied over time by regeneration, scarring, and vascular alterations, leading to an advanced stage characterized by nodularity and fibrous septation. Many of them now have specific therapies not available in the past.

Different etiologies cause different patterns of scarring and regeneration and have different rates of progression. Furthermore, within each disease entity, the magnitude of architectural distortion and the resultant clinical implications vary in severity among patients and over time. The clinical spectrum of cirrhosis is indeed wide, ranging from patients who feel no burden in their regular daily activities (a typical scenario with cirrhosis secondary to nonalcoholic steatohepatitis, for example) to severely ill patients with complications such as portal hypertension, hepatic encephalopathy, and hepatocellular carcinoma. On the other hand, there are similarities among cirrhotic livers of different etiologies extending all the way to the cellular level, reflecting common pathogenic

mechanisms: stellate cells and fibroblasts are the effectors of fibrogenesis, while parenchymal regeneration relies on hepatocytes and hepatic stem/progenitor cells.[8,9]

Until recently, fibrosis occurring in chronic liver diseases was considered a relentless process that could sometimes be halted but would not regress. However, modern treatment of chronic liver diseases has made clear that hepatic fibrosis can regress over time. Depending on the type of disease, successful treatment may involve eradication of a virus (eg, chronic hepatitis C)[10]; control of the inflammatory process following inhibition of viral replication (eg, chronic hepatitis B)[11] or suppression of autoimmunity (autoimmune hepatitis)[12]; and removal of the offending agent (eg, alcohol, iron[13]). Regression of fibrosis may take place in precirrhotic and cirrhotic livers.[5]

In recently published series regarding patients with chronic viral hepatitis undergoing treatment, there are subsets of patients with cirrhosis who show significant histologic improvement (in necroinflammatory activity and fibrosis) in repeated biopsies.[10,11,14] Although regression of cirrhosis may indeed occur in some patients,[15-17] evolution to incomplete septal cirrhosis (a condition that, despite its name, does not meet the criteria for the traditional definition of cirrhosis) has been suggested as a likely explanation for others.[5] In such cases, incomplete septal cirrhosis may result from thinning and loss of the fibrous septa surrounding the nodules (ie, regression of fibrosis), with persistence of at least some degree of the vascular changes characteristic of cirrhosis (eg, arteriovenous shunts, portal vein branch obliteration). Making this diagnosis in any particular case may have significant clinical implications, as patients with incomplete septal cirrhosis may not require orthotopic liver transplantation, as many patients with cirrhosis do, but may instead benefit from a vascular shunting procedure.

A Contemporary Approach for the Assessment of Advanced Chronic Liver Diseases

At present, there is increasing recognition of the need for a pathophysiologic staging of cirrhosis that will incorporate the clinical, histologic, and hemodynamic findings of each particular patient.[6] For example, the hepatic venous pressure gradient (HVPG) is emerging as an important parameter of a proposed classification[6] because HVPG levels have a good correlation with the complications of portal hypertension. As HVPG measurements are not widely available, surrogate histologic markers of this parameter would be desirable. For example, there is evidence that small parenchymal nodules and thick fibrous septa are associated with increased HVPG.[18,19] Therefore, the thickness of the fibrous septa has been suggested as a tool to stage advanced chronic liver

disease.[18,20] Furthermore, a significant correlation between HVPG and collagen proportionate area, a novel marker of fibrosis that can be assessed in histologic material by image analysis, has been found.[21] In addition, liver stiffness measurement, assessed by transient elastography, a noninvasive method, has been found to be in excellent correlation with HVPG values up to the level of 10 to 12 mm Hg in patients with chronic hepatitis C.[22,23]

We consider this pathophysiologic approach entirely appropriate for the present, but we suggest that it is applied to each advanced chronic liver disease independent of the term cirrhosis. For example, a pathologist examining a liver biopsy specimen from a patient with chronic hepatitis B may simply state that the patient has chronic hepatitis B of advanced stage (eg, corresponding to the descriptions for stages 5 and 6 of the Ishak staging system[24]), without using the word cirrhosis ❚Figure 1❚. The pathology report should also include the grade of necroinflammatory activity and a statement regarding risk factors for hepatocellular carcinoma (such as small cell change and large cell change), if present. This information should be sufficient for providing appropriate treatment and follow-up. Whether the advanced stage seen in the biopsy material actually represents end-stage liver disease will be determined by clinicopathologic correlation and response to treatment. Treatment adjustments can then be decided on the basis of the clinical course and subsequent biopsies, as needed.

This proposal emphasizes the etiology of liver disease in each case and has the advantage that each disease process is addressed without the negative connotations of irreversibility and end-stage nature that have traditionally been associated with the word cirrhosis. Furthermore, our approach acknowledges the fact that histologic examination alone is insufficient to document liver disease that is likely to be irreversible, while clinical assessment of advanced chronic liver disease without histologic support may not differentiate between the various causes of portal hypertension (eg, advanced chronic liver disease due to virus or steatohepatitis vs hepatoportal sclerosis). In addition, this etiology-based approach may facilitate the development of new screening strategies for early detection of hepatocellular carcinoma because there are significant differences in the incidence of this neoplasm among chronic hepatic diseases of differing etiologies.

In some cases of advanced chronic liver disease, histologic features indicative of fibrosis resorption and architectural improvement may be detected over time. The detection of histologic features suggestive of regression may represent useful information, with therapeutic and prognostic implications. Wanless et al[5] provided a detailed account of such regressive changes, which they termed "hepatic repair complex." These include delicate, perforated fibrous septa; isolated, thick collagen fibers; delicate periportal fibrous spikes; portal tract remnants; hepatic vein remnants with prolapsed hepatocytes;
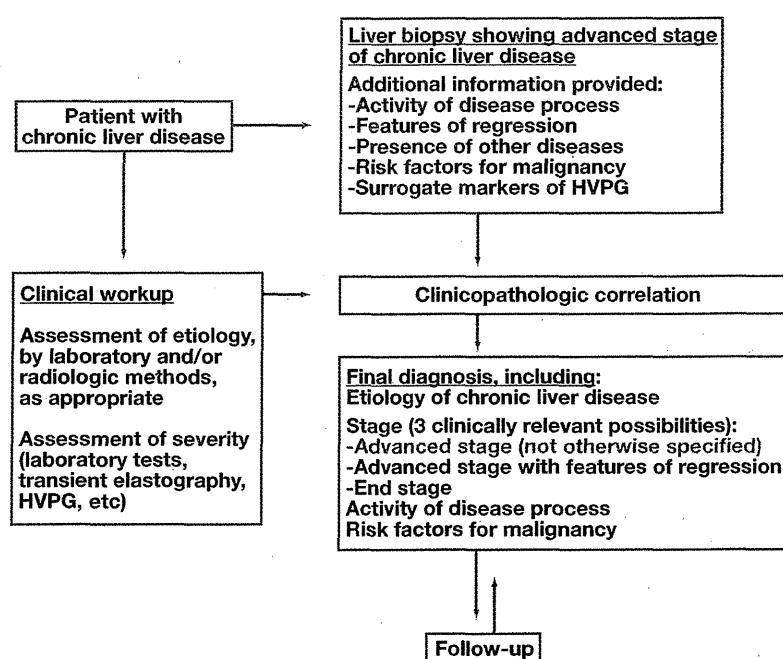

❚Figure 1❚ A contemporary approach for the assessment of advanced chronic liver diseases. The stage is determined or confirmed by liver biopsy. The final diagnosis is derived from clinicopathologic correlation (assisted by follow-up, as needed), and includes the etiology, the stage, the activity of the disease process, and risk factors for malignancy. HVPG, hepatic venous pressure gradient.

hepatocytes within portal tracts or splitting septa; minute regenerative nodules; and aberrant parenchymal veins.[5] Additional studies may further clarify the histologic features that are predictive of progression or regression of each chronic liver disease, thus aiding clinicians in patient management.

Putting Our Proposal Into Practice

Terminology changes are difficult to accomplish, especially for medical terms in widespread use, such as the word cirrhosis. Although the contemporary approach for the assessment of advanced chronic liver diseases delineated herein clearly suggests that this term has outlived its usefulness, wide consensus of pathologists and clinicians will be required for such a terminology change to become successful. This process may take some time to materialize. In the meantime, physicians involved in the diagnosis and treatment of liver diseases may explore the benefits of the recommended change.

In each patient with chronic liver disease, identification of the etiology and determination of stage are the 2 most important factors regarding prognosis and treatment. Determination of stage should be disease-specific (ie, the same staging system is not applicable for all diseases) but is often difficult to accomplish with accuracy in a small biopsy specimen, which is one more reason why a combined clinicopathologic approach seems more reasonable.[25] Keeping in mind that staging systems reflect the knowledge and the needs of the period when each of them was invented, our proposal may be considered as a starting point for a fresh look at staging chronic liver diseases in the era of regressing fibrosis.

Therefore, we recommend replacing the word cirrhosis with the term "advanced stage," when reporting the diagnosis of chronic liver diseases. Advanced stage includes cases previously diagnosed as cirrhosis, but also those with significant fibrosis and architectural distortion, which fall short of traditional cirrhosis. In cases with regression of fibrosis and architectural improvement, the term "advanced stage with features of regression" is appropriate. Assessment of fibrosis regression in biopsy material is not an easy task for practicing pathologists at present; this is best accomplished when previous biopsy specimens from the same patient are available for comparison. For cases of advanced-stage chronic liver disease with clinically significant portal hypertension, the term "end stage" may be appropriate. Obviously, carefully designed clinicopathologic studies will be needed to address the definition of end stage in each chronic liver disease. Until then, it is reasonable to associate end stage with an HVPG of 10 to 12 mm Hg or more,

representing a critical threshold beyond which chronic liver disease becomes a systemic disorder with involvement of other organs and systems.[6,26]

Some examples of how to use these diagnostic terms in pathology reporting include the following: (1) liver biopsy: autoimmune hepatitis, advanced stage, with severe activity; (2) liver biopsy: chronic hepatitis C, advanced stage, with moderate activity, and large cell change of hepatocytes; (3) liver biopsy: chronic hepatitis B, advanced stage, with features of regression, without activity; and (4) liver biopsy: nonalcoholic steatohepatitis, advanced stage (clinically, end stage), with mild activity.

Conclusion

We propose that use of the word cirrhosis should be discontinued and that each patient with chronic liver disease of advanced stage should be provided treatment on the basis of clinicopathologic correlation of all available findings (Figure 1). This proposal is consistent with our current understanding of the evolution of chronic liver diseases and will also remove an unnecessary psychological burden from patients. In addition, research into the pathogenesis and evolution of chronic liver diseases will be facilitated if an etiology-based perspective extends all the way to the end stage. In the past, ignorance was a good reason to lump the advanced stages of different liver diseases together; the opposite may prove to be more useful in the future.

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Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

Heat Shock Proteins 27 and 70 Are Potential Biliary Markers for the Detection of Cholangiocarcinoma

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Cholangiocarcinoma often is diagnosed at an advanced stage. Thus, it is necessary to establish sensitive screening methods that would allow cholangiocarcinoma and preferably its precursor lesion [biliary intraepithelial neoplasia (BilIN)] to be detected. We sought to clarify the usefulness of heat shock protein (HSP) 27 and HSP70 as biomarkers of cholangiocarcinoma and have used immunohistochemical analyses of hepatolithiatic livers to characterize HSP27 and HSP70 expression during the multistep cholangiocarcinogenesis process. HSP27 and HSP70 were measured in serum and bile samples via enzyme-linked immunosorbent assay. In hepatolithiatic tissue, the expression of HSP27 and HSP70 was increased in BilIN as well as in invasive cholangiocarcinoma. The serum levels of HSP27 and HSP70 were not significantly different between the hepatolithiatic patients with and without cholangiocarcinoma. In contrast, the bile levels of HSP27 and HSP70 were increased significantly in the patients with cholangiocarcinoma compared with those in the patients with lithiasis. Combining the measurements of the bile levels of HSP27 and HSP70 increased their usefulness as biomarkers, and the sum (HSP27 + HSP70) yielded the best sensitivity (90%) and specificity (100%). These results suggest that HSP27 and HSP70 could be used as biliary biomarkers for the detection of cholangiocarcinoma including BilIN. *(Am J Pathol 2012, 180:123–130; DOI: 10.1016/j.ajpath.2011.09.010)*

Cholangiocarcinoma often is diagnosed when it is at an advanced stage, and, hence, it displays a high mortality rate. Cholangiocarcinoma arising in the large bile ducts undergoes a multistep carcinogenesis process, and two types of precursor lesions have been proposed: biliary in-traepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB).[1–4] The former is seen in the intrahepatic large bile ducts and extrahepatic bile ducts and is classified further into three grades based on atypia: BilIN-1 (low-grade lesions), BilIN-2 (intermediate-grade lesions), and BilIN-3 (high-grade lesions, carcinoma *in situ*).

BilIN is not uncommon in the intrahepatic large bile ducts in chronic biliary diseases such as hepatolithiasis.[1–3] BilIN is a grossly unrecognizable lesion and is identifiable only on histologic sections, whereas IPNB forms a grossly visible mass, which is identifiable on radiologic images. BilIN usually is seen in the biliary epithelium around invasive cholangiocarcinoma and also incidentally is found in surgically resected specimens of hepatolithiasis. IPNB is far less common than BilIN, and the prognosis of IPNB is more favorable than that of conventional cholangiocarcinoma.[4] Considering that cholangiocarcinoma often is diagnosed at an advanced stage, it is necessary to establish sensitive screening methods that would enable cholangiocarcinoma, and preferably BilIN, to be detected.

Heat shock proteins (HSPs) are stress proteins that are inducible in response to a wide variety of insults.[5] Because they are powerful chaperones, their expression allows cells to survive otherwise lethal conditions. These cytoprotective effects are related to their ability to inhibit apoptosis.[6] HSP27 and HSP70 may participate in oncogenesis because their overexpression and the consequent inhibition of apoptosis can increase the tumorigenic potential of cancer cells. In fact, higher than normal levels of HSP27 and HSP70 were detected in cholangiocarcinoma and pancreatic cancer tissues.[7–9] HSP27 expression in cholangiocarcinoma tissues is associated with poor clinical outcome.[7] In addition, HSP27 is a potential serum marker for pancreatic cancer.[8]

To date, the temporal expression of HSP27 and HSP70 during the multistep cholangiocarcinogenesis process

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and its availability as an indicator of cholangiocarcinoma have not been studied. The present study characterized the immunohistochemical expression of HSP27 and HSP70 in the livers of hepatolithiasis patients with or without BilIN and cholangiocarcinoma, and their serum and bile levels of HSP 27 and HSP70 were analyzed to elucidate their usefulness as biomarkers.

Materials and Methods

This human study was performed with the approval of the ethics committee of Kanazawa University Graduate School of Medicine.

Tissue Preparation

In this study, hepatolithiatic livers were used as a model of the multistep cholangiocarcinogenesis process. A total of 49 hepatolithiatic livers were retrieved from the liver disease files of our laboratory and affiliated hospitals. All cases were surgically resected. Twenty-four hepatolithiatic livers were associated with BilIN and/or invasive cholangiocarcinoma in the hilar and/or perihilar region, and the remaining 25 livers were not associated with neoplastic biliary epithelial lesions. The age and sex distribution of the patients is shown in Table 1. No cases of IPNB were included in this study. As a control, the hilar regions of normal/subnormal autopsy livers ($n = 13$) were used. The samples were fixed in 10% neutral formalin and embedded in paraffin. Then, 4-μm–thick, paraffin-embedded sections were prepared. One representative section from each case was used.

Immunostaining

After deparaffinization, antigen retrieval was performed by microwaving the sections in 10 mmol/L citrate buffer (pH 6.0) for the HSP70 immunostaining. The sections then were immersed in 0.3% hydrogen peroxidase in methanol for 20 minutes at room temperature to block endogenous peroxidase activity. After pretreatment with blocking serum (DakoCytomation, Glostrup, Denmark), the

sections were incubated overnight at 4°C with primary antibodies against HSP27 (1:400, mouse monoclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and HSP70/ HSP72 (1:200, mouse monoclonal; Stressgen, Ann Arbor, MI). Then, the sections were incubated with a secondary antibody conjugated to peroxidase-labeled polymer using the HISTOFINE system (Nichirei, Tokyo, Japan). Color development was performed using 3,3′-diaminobenzidine tetrahydrochloride, and the sections were lightly counterstained with hematoxylin. Negative controls were produced by substituting the primary antibody for nonimmunized serum, which resulted in no signal detection.

Histologic Assessment

Semiquantitative analysis of the immunostained sections was performed. Staining intensity was evaluated in a high-power field for the non-neoplastic and neoplastic biliary epithelia. At least five foci were examined in each section. The signal intensity was evaluated using the following grading system: - (negative), 1+ (mild to moderate), 2+ (marked).

Enzyme-Linked Immunosorbent Assay

The HSP27 and HSP70 levels of serum and bile samples were measured using enzyme-linked immunosorbent assay. The serum samples were obtained from 56 patients with hepatolithiasis, eight of whom had clinically detectable cholangiocarcinoma in the hilar region of their liver. Among these eight cholangiocarcinoma cases, surgical resection was performed in four cases, which resulted in noncurative resection, and the other four cases were not indicated for surgical resection because of disease progression. As controls, serum samples obtained from 16 healthy volunteers were used.

The bile samples were obtained from patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$) and patients with cholangiocarcinoma (without lithiasis) ($n = 10$). The cholangiocarcinoma cases without lithiasis consisted of hilar ($n = 6$) and extrahepatic ($n = 4$) cholangiocarcinoma, and all of these cases presented with obstructive jaundice. The bile samples were obtained by percutaneous transhepatic cholangiographic drainage. The age and sex distributions of the patients from whom the serum and bile samples were obtained are summarized in Table 1.

HSP27 and HSP70 levels were measured using the HSP27 enzyme-linked immunosorbent assay kit (Stressgen) and the HSP70 High Sensitivity EIA Kit (Stressgen), respectively, according to the manufacturer's instructions. Briefly, samples were added to a 96-well plate coated with a monoclonal antibody against HSP27 or HSP70 and incubated for the time indicated in the manufacturer's protocol at room temperature. After being washed, the plate was incubated with anti-HSP27 or anti-HSP70 antibody conjugated to horseradish peroxidase for 1 hour at room temperature. Color development was performed using a substrate solution, and absorbance was measured at 450 nm.

Table 1. Age and Sex Distribution of the Cases Studied

Specimen	n	Age (years)	Sex (M:F)
Tissue			
Normal/subnormal liver	13	62 ± 7	6:7
Hepatolithiasis	25	55 ± 8	8:17
Cholangiocarcinoma with hepatolithiasis	24	59 ± 10	7:17
Serum			
Control	16	60 ± 15	10:6
Hepatolithiasis	48	74 ± 10	26:22
Cholangiocarcinoma with hepatolithiasis	8	76 ± 10	4:4
Bile			
Lithiasis	10	61 ± 15	5:5
Cholangiocarcinoma without lithiasis	10	76 ± 10	6:4

F, female; M, male.

Hepatolithiasis

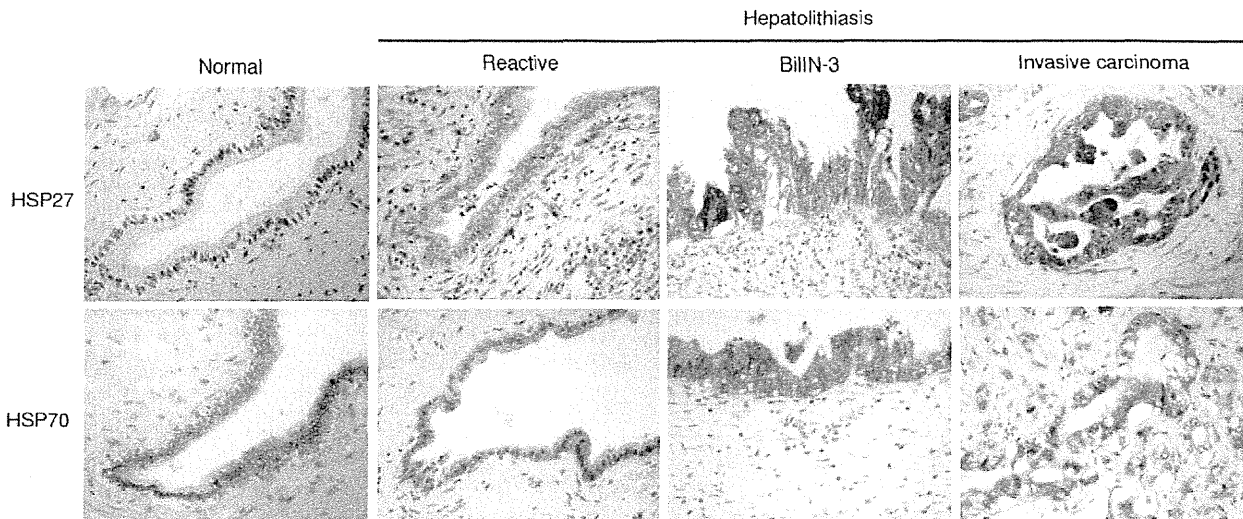| Normal | Reactive | BilIN-3 | Invasive carcinoma |

Figure 1. Immunohistochemical expression of HSP27 and HSP70 in hepatolithiasis. The immunohistochemical expression of HSP27 and HSP70 was faint and weak, respectively, in the biliary epithelium of the large bile ducts of normal livers. In hepatolithiasis, the expression of HSP27 and HSP70 tended to be intense in BilIN and invasive cholangiocarcinoma than in the non-neoplastic, reactive biliary epithelium. Original magnification, ×400.

Statistics

The data are expressed as the mean ± SD. Statistical significance was determined using the Mann–Whitney U test and the χ^2 test using Statview-J5.0 software (Abacus Concepts, Inc., Berkley, CA). P values of <0.05 were accepted as statistically significant. Receiver operating characteristic curves were constructed by plotting sensitivity versus 1 − specificity using Dr SPSS II software (version 11.01 J; SPSS Japan, Inc., Tokyo, Japan), and the area under the curve was calculated.

Results

Immunohistochemical Expression of HSP27 and HSP70

The results of immunohistochemical staining for HSP27 and HSP70 are shown in Figure 1. In the vast majority of normal livers, the immunohistochemical staining of HSP27 was faint or negligible, and that of HSP70 was weak in the biliary epithelium of the large bile ducts. Hepatocytes lacked positive signals for HSP27 and HSP70. Vascular smooth muscle cells, nerve fibers, and

the microvessel endothelium constitutionally expressed HSP27, and the biliary epithelium of the small bile ducts also was positive for HSP70.

In hepatolithiasis, the epithelia of the large bile ducts and the neoplastic biliary epithelium showed various degrees of HSP27 and HSP70 expression (Figure 1). HSP27 was observed in the cytoplasm, whereas HSP70 was localized in the cytoplasm and/or the nuclei. Their expression tended to be intense in BilIN and invasive cholangiocarcinoma compared with that in the non-neoplastic, reactive biliary epithelium.

Semiquantitative Analysis of HSP27 and HSP70 Expression

The signal intensity of the immunohistochemical expression of HSP27 and HSP70 in the biliary epithelium was categorized into three grades, and a semiquantitative analysis was performed. The expression levels of HSP27 and HSP70 tended to be high in BilIN and invasive cholangiocarcinoma compared with those in the non-neoplastic biliary epithelium of the hepatolithiasis patients and the normal bile ducts (Figure 2, B and C).
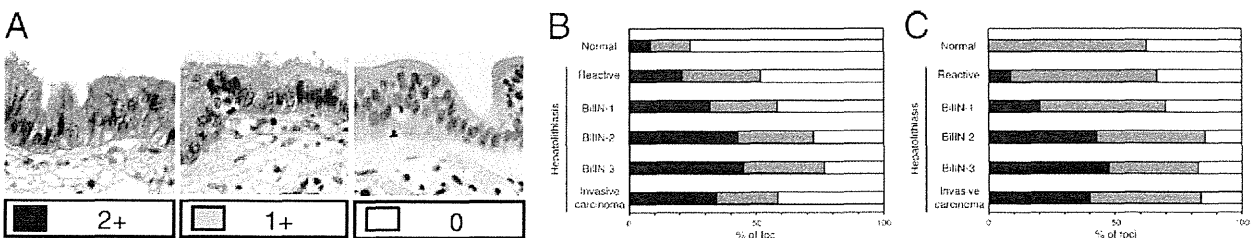

Figure 2. Semiquantitative analysis of the immunohistochemical expression of HSP27 and HSP70. **A:** The intensities of the immunohistochemical signals for HSP27 and HSP70 in the epithelium were categorized into three grades. Semiquantitative analysis was performed as described in *Materials and Methods*. The expression levels of both HSP27 (**B**) and HSP70 (**C**) tended to be higher in BilIN and invasive cholangiocarcinoma than in the non-neoplastic biliary epithelia of the hepatolithiasis patients and the normal bile ducts.
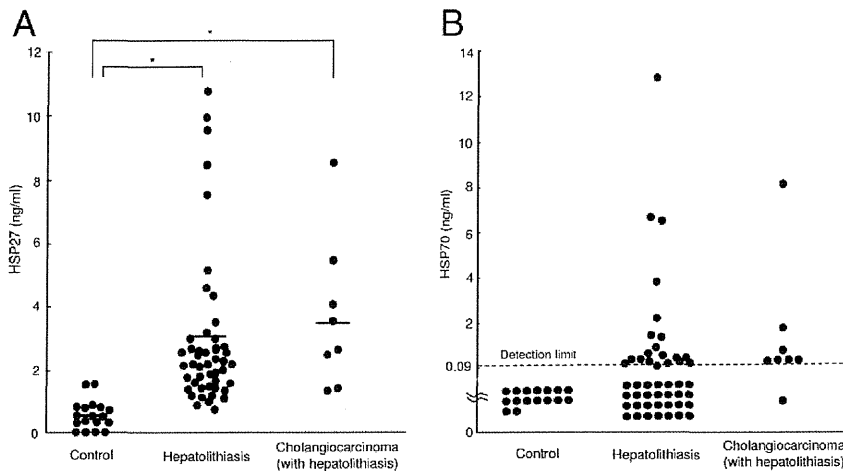
Figure 3. Serum HSP27 and HSP70 concentrations. The concentrations of HSP27 (**A**) and HSP70 (**B**) in serum were determined in healthy controls ($n = 16$) and patients with hepatolithiasis without cholangiocarcinoma ($n = 48$) and patients with hepatolithiasis with clinically detectable cholangiocarcinoma ($n = 8$). The hepatolithiasis patients with or without cholangiocarcinoma showed significantly higher serum levels of HSP27 than the healthy controls, but the difference between the hepatolithiatic patients with and without cholangiocarcinoma was not significant. None of the healthy control serum samples contained detectable amounts of HSP70 (detection limit, 0.09 ng/mL). Of the hepatolithiasis patients without cholangiocarcinoma, 20 displayed serum HSP70 levels that exceeded the detection limit. Of the cholangiocarcinoma patients with hepatolithiasis, seven showed serum HSP70 values that were above the detection limit. **A:** The black bars indicate mean values. *$P < 0.01$.

Serum HSP27 and HSP70 Concentrations

The serum concentrations of HSP27 were 0.55 ± 0.49, 3.03 ± 2.52, and 3.71 ± 2.39 ng/mL in the healthy controls ($n = 16$) and the patients with hepatolithiasis without cholangiocarcinoma ($n = 48$), and patients with hepatolithiasis with clinically detectable cholangiocarcinoma ($n = 8$), respectively (Figure 3A). Statistical analysis showed that the patients with hepatolithiasis with or without cholangiocarcinoma showed significantly higher serum levels of HSP27 than healthy controls, but the difference between the hepatolithiatic groups with and without cholangiocarcinoma was not significant.

None of the healthy controls ($n = 16$) displayed detectable amounts of HSP70 in their serum, according to measurements taken using a commercially available EIA Kit (detection limit, 0.09 ng/mL; Stressgen) (Figure 3B). Of the hepatolithiasis patients without cholangiocarcinoma ($n = 48$), 20 displayed serum HSP70 levels that exceeded the detection limit, whereas the values of the other 28 cases remained below the detection limit (pos-

itive detection rate, 41.7%). Among the patients with cholangiocarcinoma with hepatolithiasis ($n = 8$), seven cases displayed serum HSP70 values that were above the detection limit (positive detection rate, 87.5%). Although the positive detection rate was significantly higher in the cholangiocarcinoma patients than in the hepatolithiasis patients without cholangiocarcinoma ($P < 0.05$), their HSP70 levels were not significantly different.

In four cases of hepatolithiasis, serum samples were available before and after the development of cholangiocarcinoma. In these four cases, the patients' serum HSP27 levels tended to decrease after the development of cholangiocarcinoma (Figure 4A), whereas their serum HSP70 levels were increased or unchanged after they developed cholangiocarcinoma (Figure 4B).

Bile HSP27 and HSP70 Concentrations

Bile samples were obtained from patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$) and chol-
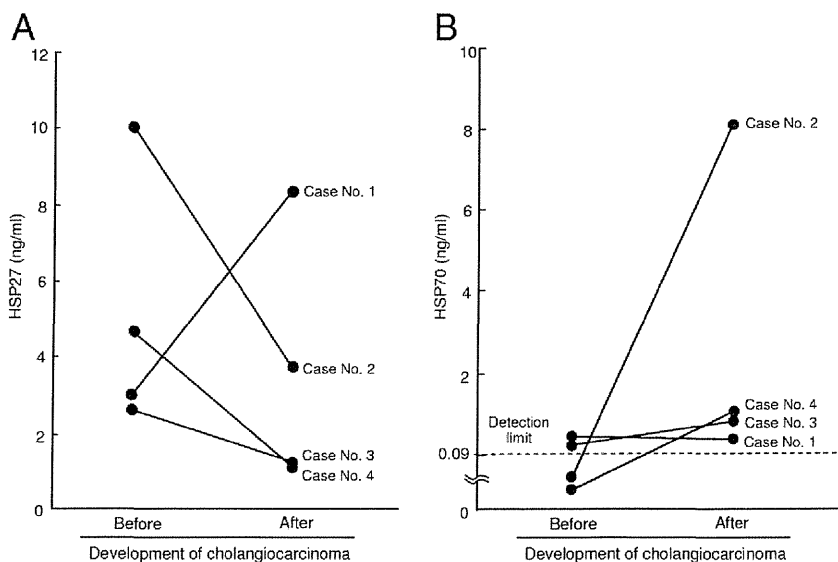

Figure 4. Comparison of serum HSP27 and HSP70 concentrations before and after the development of cholangiocarcinoma. In four cases of hepatolithiasis, serum samples were obtained before and after the development of cholangiocarcinoma, and their values of HSP27 (**A**) and HSP70 (**B**) were compared. Their serum HSP27 levels tended to decrease after the development of cholangiocarcinoma, whereas their serum HSP70 levels were increased or unchanged after the development of cholangiocarcinoma.
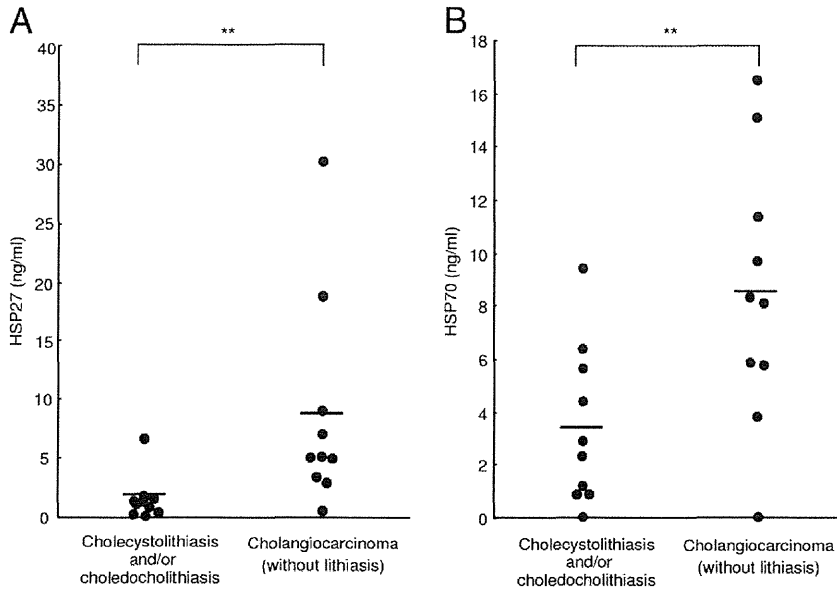
Figure 5. Bile HSP27 and HSP70 concentrations. The bile levels of HSP27 (**A**) and HSP70 (**B**) were examined in samples obtained from patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$), and patients with cholangiocarcinoma (without lithiasis) ($n = 10$). For both HSP27 and HSP70, the measured values were significantly higher in the cholangiocarcinoma patients than in the patients with cholecystolithiasis and/or choledocholithiasis. The black bars indicate mean values. **$P < 0.05$.

angiocarcinoma patients without lithiasis ($n = 10$), and the HSP27 and HSP70 levels of these samples were examined. The HSP27 bile levels of the former and latter groups were 1.48 ± 1.80 and 8.52 ± 8.97 ng/mL, respectively, and a significant difference was observed between them (Figure 5A).

The bile HSP70 concentrations of the patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$) and cholangiocarcinoma without lithiasis ($n = 10$) were 3.41 ± 3.04 and 8.39 ± 4.98 ng/mL, respectively, and a significant difference was observed between them (Figure 5B). The bile HSP27 and HSP70 levels of the cholecystolithiasis and/or choledocholithiasis (Figure 6A) and cholangiocarci-

noma without lithiasis patients (Figure 6B) showed almost parallel distributions, with several exceptions.

When the product and the sum of the values of HSP27 and HSP70 were calculated, both the product (HSP27 × HSP70) and sum (HSP27 + HSP70) were significantly higher in the cholangiocarcinoma patients without lithiasis than in those with cholecystolithiasis and/or choledocholithiasis (Figure 7, A and B).

A receiver operating characteristic curve was constructed for the bile HSP27 and HSP70 measurements. The resultant analysis showed that the sum of HSP27 and HSP70 was the best indicator of cholangiocarcinoma, producing a sensitivity of 90% and a specificity of 100%
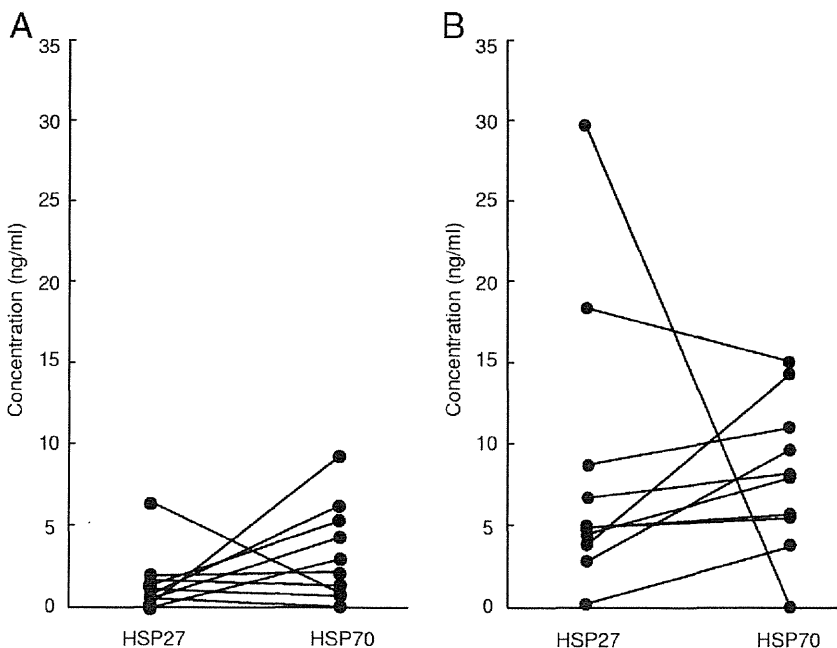

Figure 6. Comparison of bile HSP27 and HSP70 concentrations. The bile levels of HSP27 and HSP70 were compared between the patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$) (**A**), and the patients with cholangiocarcinoma (without lithiasis) ($n = 10$) (**B**). The patients' bile levels of HSP27 and HSP70 showed an almost parallel distribution in both groups with several exceptions.
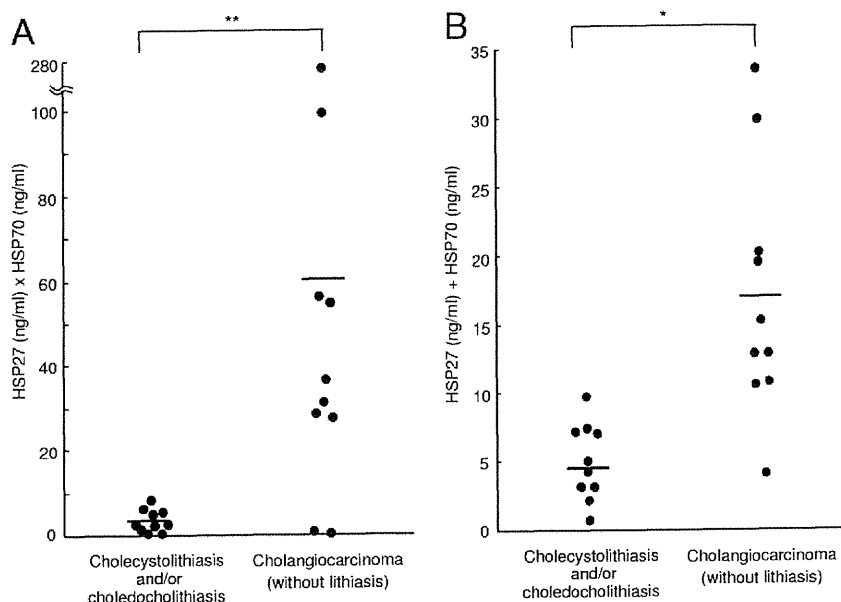
Figure 7. Analysis of the data on bile HSP27 and HSP70 concentrations. The product (**A**) and sum (**B**) of the bile HSP27 and HSP70 levels were calculated for the patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$), and the patients with cholangiocarcinoma (without lithiasis) ($n = 10$). Both the product (HSP27 × HSP70) and the sum (HSP27 + HSP70) were significantly higher in the cholangiocarcinoma patients than in the patients with cholecystolithiasis and/or choledocholithiasis. The black bars indicate mean values. $^*P < 0.01$; $^{**}P < 0.05$.

at a cut-off value of 10.2 (Table 2). The area under the curve was calculated as 0.940.

Discussion

This study showed that HSP27 and HSP70 could be used as biliary markers for the detection of cholangiocarcinoma. Combining the bile values of HSP27 and HSP70 further increased their usefulness as biomarkers, and the sum (HSP27 + HSP70) yielded the best sensitivity (90%) and specificity (100%). In contrast, the levels of HSP27 and HSP70 in serum were not significantly different between the groups with and without cholangiocarcinoma. Immunohistochemical analysis showed that the expression of HSP27 and HSP70 was increased in BilIN as well as cholangiocarcinoma, indicating that the increased HSP27 and HSP70 bile levels found in these patients were caused by the local production and secretion of these molecules by the neoplastic epithelium.

Biliary tumor markers are considered to be secreted from bile and into the serum, possibly as a result of increasing biliary pressure owing to local obstruction triggered by the loss of cellular polarity.[10] Therefore, the bile levels of HSP27 and HSP70 might reflect more accurately the local production of these molecules in the biliary tract than their serum levels.

The diagnostic values of the bile levels of carbohydrate antigen 19-9 and carcinoembryonic antigen have been investigated previously.[11–18] The frequency of carbohydrate antigen 19-9 detection in bile from patients with both benign and neoplastic pancreaticobiliary tract diseases has been reported to range from 46% to 61%,[13,14] and the associated specificity has been reported to range from 60% to 70%.[15] Increased bile carcinoembryonic antigen levels also have been shown to predict cholangiocarcinoma with a sensitivity of 58% to 84% and a specificity of 33% to 84%.[14–17] However, factors such as the presence of cholangitis, infection, and the sampling time (ie, before or after biliary drainage) have been shown to significantly influence the levels of carbohydrate antigen 19-9 and carcinoembryonic antigen and so the diagnostic value of bile carbohydrate antigen 19-9 and carcinoembryonic antigen levels is disputed.[13,17,18]

In this study, it was unclear whether the patients without cholangiocarcinoma and/or BilIN had other disorders such as cholecystitis and pancreatitis. It should be noted that the absence of patients with these conditions could have increased the specificity of these markers and improved the area under the receiver operating characteristic curve. This was a potential limitation of the current study, which further studies will have to address.

Recently, *Wisteria floribunda* agglutinin–positive mucin 1 was identified as a novel biliary marker of cholangiocarcinoma that enables cholangiocarcinoma to be distinguished from benign diseases with a sensitivity of 90.0% and a specificity of 76.3%.[19] The sensitivity of the bile HSP27 + HSP70 level in this study (90%) was comparable with that found in their study, but the specificity of the bile HSP27 + HSP70 level (100%) exceeded that of their study. More recently, it was shown that bile proteomic profiles can differentiate cholangiocarcinoma from benign biliary diseases.[20] Thus, simultaneously testing the

Table 2. Receiver Operating Characteristic Curve Analysis of HSP27 and HSP70 Bile Concentrations as Predictors of Cholangiocarcinoma

	Cut-off value	Sensitivity (%)	Specificity (%)	AUC
HSP27 (ng/mL)	2.52	90	90	0.860
HSP70 (ng/mL)	5.67	80	80	0.805
HSP27 × HSP70	17.9	80	100	0.825
HSP27 + HSP70	10.2	90	100	0.940

Bile samples from cholangiocarcinoma ($n = 10$) and lithiasis patients ($n = 10$) were analyzed.
AUC, area under the curve.

bile levels of several markers seems to be more useful for detecting cholangiocarcinoma.

A previous study showed that serum HSP27 was a useful marker for the detection of pancreatic cancer, displaying a sensitivity of 100% and a specificity of 84%.[8] However, in this study, higher serum HSP27 levels were observed in patients with hepatolithiasis with or without cholangiocarcinoma than in the healthy controls, but the difference between the hepatolithiatic patients with and without cholangiocarcinoma was not significant. Similarly, the serum HSP70 levels of the hepatolithiatic patients with and without cholangiocarcinoma were not significantly different, but the positive detection rate was significantly higher among the patients with cholangiocarcinoma than among the hepatolithiasis patients without cholangiocarcinoma. Thus, the serum HSP70 levels could be a useful screening tool that could lead to further evaluations.

As shown in Figure 2, the immunohistochemical analysis showed that the expression levels of HSP27 and HSP70 were increased in the non-neoplastic, reactive biliary epithelia of several hepatolithiasis patients compared with those in the normal biliary epithelium. These results suggest that cholangitis caused by hepatolithiasis is associated with the induction of HSP27 and HSP70 expression in the biliary epithelium and might account for the increased serum HSP27 and HSP70 levels of the patients without clinically detectable cholangiocarcinoma. In addition, the possibility that hepatolithiatic patients with BilIN were included in the experimental groups (hepatolithiasis without cholangiocarcinoma) of the serum enzyme-linked immunosorbent assay study cannot be excluded because BilIN is a clinically unrecognizable lesion.

Recent studies have identified several epigenetic and genetic factors that are involved in the multistep cholangiocarcinogenesis process in hepatolithiasis. For example, p16, p21, p53, cyclin D1, β-catenin, and E-cadherin have been implicated in the process of carcinogenesis.[21–24] The present study showed that HSP27 and HSP70 also are involved in the cholangiocarcinogenesis. HSP27 and HSP70 inhibit carcinoma cell apoptosis, and their overexpression is associated with resistance to treatment.[25–27] The tumorigenic role of Fas/FasL in cholangiocarcinoma has been described, and it is suggested that this pathway is a potential molecular target of therapeutic strategies that aim to circumvent apoptosis resistance in cholangiocarcinoma.[28] Although the mechanisms that regulate HSP27 and HSP70 expression have not been fully defined, they are potential targets of cholangiocarcinoma therapy.

In summary, this study showed that the expression of HSP27 and HSP70 was induced in BilIN as well as invasive cholangiocarcinoma, indicating that their induction is an early event in the development of cholangiocarcinoma and is associated closely with the multistep carcinogenesis process. Accordingly, the bile levels of HSP27 and HSP70 were significantly higher in patients with cholangiocarcinoma than in the control subjects, suggesting that HSP27 and HSP70 could be used as biliary biomarkers for the detection of cholangiocarcinoma. Because the expression of HSP27 and HSP70 was induced in BilIN, they also might be applicable to the detection of BilIN. These findings are preliminary, and a prospective trial needs to be conducted to determine the performance characteristics of an assay using these markers in clinical practice.

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Review Article

Tutorial Review for Understanding of Cholangiopathy

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The biliary tree consists of intrahepatic and extrahepatic bile ducts and is lined by biliary epithelial cells (or cholangiocytes). There are also peribiliary glands around the intrahepatic large bile ducts and extrahepatic bile ducts. The biliary tree is a conduit of bile secreted by hepatocytes and biliary epithelial cells and also of the peribiliary glands and has several physiological roles. A number of diseases affect mainly the intrahepatic and extrahepatic biliary tree, and, in this special issue, these cholangiopathies are reviewed in detail with respect to genetics, pathogenesis, and pathology. In this paper, the anatomy and physiology of the biliary tree, basic injuries to biliary epithelial cells from stress and bile duct damage, and representative cholangiopathies are briefly reviewed.

1. Introduction

A number of diseases affect the biliary tree (cholangiopathies), though the pathological mechanisms involved and the anatomical level of the biliary tree affected vary [1]. For example, small interlobular bile ducts are mainly affected by a Th1-dominated microenvironment and cell-mediated immune response in PBC [2], while a Th2-dominated microenvironment and increased numbers of regulatory T cells are the major features of IgG4-related sclerosing cholangitis which affects mainly the extrahepatic bile ducts [3]. Ischemic damage to the biliary tree is a serious complication in liver transplantations [4].

In this special issue, cholangiopathy with respect to genetics, pathogenesis, and pathology will be discussed in detail. Herein, the anatomy and physiology of the biliary tree, basic injuries to biliary epithelial cells, basic forms of bile duct damage, and etiological classifications of cholangiopathy are reviewed. This tutorial review will be helpful for a better understanding of cholangiopathy.

2. Anatomy and Characteristics of the Biliary Tree

2.1. Anatomy. The biliary tree is composed of extrahepatic and intrahepatic bile ducts [5]. The former include the right and left hepatic ducts and their confluence and the common hepatic and bile ducts, while the latter include the bile ducts proximal to the right or left hepatic duct. The intrahepatic branching of the bile ducts is best visualized on a cholangiograph or biliary injection cast (Figures 1 and 2). The extrahepatic bile duct is lined by high columnar epithelial cells, and its wall is composed of dense collagenous tissue harboring scattered smooth muscular elements.

The intrahepatic bile ducts can be classified as large and small, though there is no sharp delineation of the various segments [1, 5]. The biliary epithelial cells or cholangiocytes compose approximately 4-5% of liver mass. The large type consists of the right and left hepatic bile ducts and their first to third branches (segmental and area bile ducts). These ducts are grossly visible and belong to the perihilar bile ducts. They are lined by a tall columnar epithelium and surrounded by a dense hypocellular collagenous duct wall. In contrast, small intrahepatic bile ducts, the branches of the large intrahepatic bile duct, are classified into septal and interlobular bile ducts which are visible only under a microscope. While the septal ducts (>100 μm in diameter) are lined by tall columnar cells with basal nuclei, the interlobular bile ducts are lined by cuboidal cells. The fibrous ductal wall is evident in the former like large intrahepatic ducts, but not in the latter. The interlobular bile ducts are connected to the bile canalicular network by ductules (<20 μm diameter) lined by no more than a few minimally differentiated cuboidal cells and the canals of Hering, which are lined partly by biliary
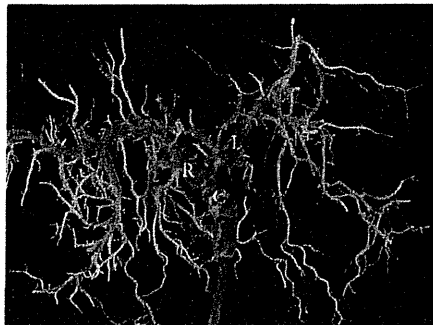
FIGURE 1: Biliary cast of normal liver. C: common hepatic duct, L: left hepatic duct, and R: right hepatic duct.

FIGURE 3: Peribiliary glands. B: bile duct; arrows: peribiliary glands and their conduits.
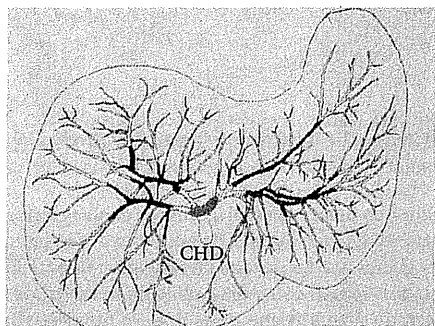

FIGURE 2: Diagram of the biliary tree. Red: right and left hepatic duct and their confluence, yellow: branches of the right or left hepatic ducts, and black: further branches. CHD: common hepatic duct.

epithelium and partly by hepatocytes. The intrahepatic stem cell niches are the canals of Hering in postnatal livers [6]. Bile ductules are very reactive anatomical elements in the liver, and proliferating bile ductules are reportedly involved in the fibrous progression of various chronic liver diseases and are easily identifiable by immunostaining of biliary cytokeratin (CK 7 and 19).

Peribiliary glands, the third biliary component, are present within the fibromuscular walls of extrahepatic bile ducts and also along the large intrahepatic bile ducts [1, 5, 7]. Glandular elements are also found at the neck of the gallbladder. Peribiliary glands around the large intrahepatic bile ducts (Figure 3) are subdivided into intramural glands, nonbranching tubular glands, and extramural ramified glands. The latter lie in the periductal connective tissue and, in a three-dimensional model, have a linear distribution along the opposite sides of the bile ducts and indirectly drain into the bile duct lumen via their own conduit. The extramural glands consist of serous and mucous acini. Pancreatic acini without Langerhans' islets are found intermingled with peribiliary glandular acini and are probably an intrinsic component of these glands. The glands are thought to have secretory activities. The extrahepatic stem cell niches are the peribiliary glands deep within the walls of the bile duct [6, 8].

2.2. Distribution of Antigens along the Biliary Tree.

The individual anatomical components of the biliary tree each have a rather characteristic antigen, probably reflecting a site-specific function [9, 10]. For example, the BECs lining large bile ducts are columnar and mucus is detectable in the supranuclear cytoplasm, but mucin is not detectable in the interlobular bile ducts and bile ductules. In contrast, in the adult liver, the BECs of intrahepatic large bile ducts constantly express MUC3, a membrane-binding type, whereas those of small bile ducts do not. MUC6 is constantly and focally expressed in BECs in the intrahepatic large bile ducts in normal liver. The expression of MUC1, MUC2, and MUC5 was infrequent in normal livers but increased in hepatolithiasis [9]. This study disclosed that the normal biliary tree has a specific expression of blood group antigens at different levels and that this expression is altered under pathologic conditions. In normal livers, large and septal bile ducts expressed A and B antigens in patients with comparable blood groups and also expressed H antigen frequently in patients with blood group O, A, or B and infrequently in patients with type AB. Lea and Leb are expressed in BECs at any level in secretors. As for cytokeratin, CK7 and CK19 are expressed in BECs of the biliary tree and also in peribiliary glands, while EpCAM is expressed in bile ductules. CK8 and 18 are expressed in hepatocytes and also BECs of the biliary tree.

2.3. Supply of Blood to the Biliary Tree.

The intrahepatic and extrahepatic biliary tract is supplied by a network of fine vessels called the peribiliary vascular plexus (PBP) which exclusively derives from hepatic arterial branches [11–13]. The PVP can be histologically divided into the inner, intermediate, and outer layers, with respect to the bile duct walls [12]. These three layers are well and poorly developed in the large intrahepatic bile ducts and septal bile ducts, respectively, although the PBP around the interloblar bile ducts and bile ductules consists of scattered capillaries with no discernible layers [13]. This plexus has a fern-like appearance around the bile duct under the scanning electron microscope. The PBP drains into the sinusoids through "radicular portal veins" or communicates with portal venous

branches through "internal roots" or directly into the hepatic sinusoids in animals and probably in humans.

The inner layer, a layer of capillaries, is found just beneath the basement membrane of the epithelial layer and is regularly distributed like a chain. Ultrastructurally, the inner capillary layers are composed of fenestrated endothelial cells, and the number of fenestrae with a thin diaphragm is rather high on the capillary side facing the bile duct epithelium [13]. These observations suggest that the PBP, particularly the inner layer, may participate in the physiology of the bile ducts, particularly in the exchange of substances between blood in the peribiliary vascular plexus and bile in the bile ducts and in the supply and drainage of substances to and from the biliary epithelia [12].

2.4. Physiological Roles of the Biliary Tree.

The biliary tree is lined by specialized epithelial cells called BECs or cholangiocytes [14] and is not only a conduit of bile secreted by hepatocytes and cholangiocytes but also a conduit of the peribiliary glands. The bile ducts and peribiliary glands play a number of physiological roles in the biliary system, contributing to about one-third of total bile secretion, participating in bile acid and water reabsorption, and secretion via transporters, and also mediating immune responses including innate immunity [15]. The primary hepatic bile secreted by hepatocytes is modified by BECs via a series of secretory and absorptive processes that provide additional bile water (BECs secrete ~40% of daily bile production in humans) or secrete HCO_3^- to induce an alkakine state [14]. BECs also interact with the immune system and microorganisms and are also involved in drug metabolism. To accomplish these functions, BECs display morphological and functional heterogeneity along the biliary tree.

2.5. Innate Immunity.

The biliary tree is essentially sterile under normal conditions, but bile is potentially contaminated by bacterial components such as pathogen-associated molecular patterns (PAMPs) including lipopolysaccharide (LPS) and bacterial DNA originating from intestinal flora, which are actually detectable in bile of patients with chronic inflammatory biliary diseases [16]. In this context, the biliary tract is equipped with defence mechanisms, which are physical (bile flow and biliary mucus), chemical (bile salts), and immunological, such as secretory IgA. BECs also express Toll-like receptors (TLR) and intracellular adaptor molecules and secrete antibiotic peptides and (pro)inflammatory cytokines, thereby participating in the defense of the bile ducts [15].

Nonspecific bactericidal enzymes such as lactoferrin and lysozyme are also detected in the intrahepatic biliary tree, peribiliary glands, and bile [15]. Human β-defensins (hBDs) and cathelicidin, another antimicrobial peptide contributing to innate immunity at mucosal surfaces, are expressed in the biliary tree. hBD-1 is constitutively expressed in the biliary epithelium, while hBD-2 is expressed in large intrahepatic bile ducts in extrahepatic biliary obstruction, hepatolithiasis, and, to a lesser degree, PBC and PSC, suggesting a response to local infection or bacterial components, cytokines such as

IL-1β and TNF-α, and/or active inflammation. Cathelicidin is expressed by normal biliary epithelial cells in addition to hepatocytes. Trefoil factor family (TFF) 1, 2, and 3 peptides expressed at the apical surface of the epithelium play a major role in mucosal repair.

IgA is known to be secreted into bile by binding with the secretory component (SC), and secretory IgA (SIgA) functions in a number of ways to protect the biliary tract. For example, it can directly bind and neutralize bacterial toxins. SIgA can bind to bacteria and prevent their adhesion to the mucosal membrane. Additionally, IgA has been demonstrated to neutralize intracellular microbes and their products. Biliary intraepithelial lymphocytes (bIELs), which are markedly increased in immune-mediated cholangitis, are occasionally encountered in normal intrahepatic bile ducts. Most of them are positive for CD8, some are positive for CD57, and these cells may participate in biliary innate immunity [17].

3. Basic Injuries of Biliary Epithelial Cells and Bile Duct Damage

3.1. Basic Injuries of BECs.

Several pathologic agents and stress affect the intrahepatic and extrahepatic biliary tree including viral, bacterial, and even parasitic infections, oxidative stress, and immunological assaults, as well as biliary epithelial injuries from necrosis, apoptosis, and hyperplasia, and also bile duct damages.

3.1.1. Apoptosis and Necrosis.

In some biliary diseases such as primary biliary cirrhosis (PBC) and chronic ductopenic allograft rejection, the ongoing apoptosis of BECs is important for progressive bile duct loss. In H&E stained sections, eosinophilic, shrunken slender cells with pyknotic nuclei in the biliary epithelial layer and fragmented and condensed nuclei in the bile duct lumen can be regarded as apoptotic bodies [2, 15]. Electron microscopically, shrunken BECs with a condensed cytoplasm and pyknotic nuclei are a marker of apoptosis. Apoptosis of BECs can be confirmed using in situ nick-end labelling and immunostaining of single stranded DNA, both of which detect DNA fragmentation. In contrast, the coagulative or lytic necrosis of the biliary epithelium is occasionally encountered in toxic cholangiopathy [18].

3.1.2. Cellular Senescence.

Senescent BECs show characteristic features such as an eosinophilic cytoplasm, cellular and nuclear enlargement, multinucleation, and an irregular arrangement with uneven nuclear spacing [19]. Actually, these cells also express cellular senescent markers such as the cell cycle regulators, p16^{INK4} and p21$^{WAF1/CIP}$, and increased activity of senescence-associated β-galactosidase (SA-β-gal). Recent studies showed that cellular senescence has at least two pathological effects in the development of biliary diseases: impaired regeneration and senescence-associated secretory phenotypes (SASPs).

Impaired Regeneration. Senescent cells no longer have the ability to proliferate and they are irreversibly arrested at