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Cholangiocarcinoma in primary sclerosing cholangitis

by
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Abstract

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease of unknown causes closely associated with ulcerative colitis. PSC is a progressive disease leading to liver failure and need for liver transplantation. Cholangiocarcinoma (CC) occurs in 10 – 20% of patients with PSC. The prognosis for CC is poor, even after liver transplantation. It is of great importance to identify PSC patients at risk for malignant development and transplant them at an early stage. Tools for early diagnosis of CC and possibilities to detect pre-malignancy are lacking. The general purpose of this thesis was therefore to identify early diagnostic markers and risk factors for malignancy in PSC.

The first study was a case-control study comparing 20 PSC patients with CC and 20 patients with end stage PSC without cancer, the aims were to assess and compare clinical features in these groups and identify risk factors for the development of cancer. No difference was found in clinical presentation, laboratory or radiological findings. The number of patients being either current or former smokers was significantly higher in the cancer group than among controls ($p < 0.0004$). To analyse the concept of bile duct dysplasia and the possibility of agreement of this morphological feature and determine reproducibility of the diagnosis, livers from 26 PSC patients with and 60 without concomitant CC were studied. Criteria for bile duct dysplasia were defined with reasonable level of agreement among three hepatopathologists, the kappa level for dysplasia being 0.44. Comparison of the frequency of bile duct dysplasia in livers from patients with PSC with and without CC showed dysplasia in 19% (5/26) of the cancer patients and in 0% (0/60) of non-cancer patients ($p < 0.001$). In CCs and in non-tumourous liver tissue from 16 PSC patients with and 16 patients without CC, bile duct cell proliferation, apoptosis and expression of p53 and bcl-2 proteins were studied. Histological stage, presence of bile duct dysplasia and immunohistochemical staining for Ki-67, nuclear DNA fragmentation, p53 and bcl-2 in non-tumourous liver tissue from PSC patients with and without CC did not differ significantly. Patients with bile duct dysplasia ($n=9$) had a significantly higher frequency of moderate/marked bile duct proliferation than those without bile duct dysplasia ($p < 0.01$). In addition, evaluation of the ploidy of DNA in CCs from patients with and without PSC was made. CCs from patients with PSC displayed DNA aneuploidy significantly more often (8/10) than CCs from patients without PSC (7/18) ($p < 0.05$). 12% (2/17) of large bile ducts from PSC patients without CC displayed aneuploidy of DNA. In a large cohort of Swedish PSC patients ($n=604$), we assessed the risk of malignancies in PSC compared to the general Swedish population. The frequency of hepatobiliary malignancies was 13.3%. The standardized incidence rate for hepatobiliary carcinoma was 161, and 14 for pancreatic carcinoma.

In conclusion, it is difficult in clinical settings to distinguish PSC patients with end stage disease from those with liver malignancy. PSC patients being current or former smokers are at an increased risk of developing hepatobiliary carcinoma. Criteria for bile duct dysplasia can be agreed on and the entity recognised in liver biopsies. The strong association of biliary dysplasia with cholangiocarcinoma in PSC suggests that occurrence of dysplasia can be used as a marker for current or developing malignancy. Increased bile duct proliferation may be used as a surrogate marker for premalignancy in PSC. The majority of CCs in PSC display DNA-aneuploidy. PSC patients also run an increased risk of developing pancreatic carcinoma.

Keywords: Primary sclerosing cholangitis, pancreatic carcinoma, cholangiocarcinoma, ulcerative colitis, biliary dysplasia, apoptosis, Ki-67, p53, bcl-2.

*“Det finns världsallt, solar och atomer.
Det finns en kunskap, strategiskt byggd på fasta punkter.
Det finns en kunskap, oförsvard byggd på osäkert tomrum.
Det finns ett tomrum mellan världsallt, solar och atomer.
(Vad rör mig världsallt, solar och atomer.)
Det finns den udda synpunkten på allt i detta dubbla liv.”*

Gunnar Ekelöf, 1961

Till Mattis, Erik and Axel

Abbreviations

CC – cholangiocarcinoma

CD – Crohn's disease

CT – computer tomography

ERCP – endoscopic retrograde cholangiopancreatography

GBC – gallbladder carcinoma

HCC – hepatocellular carcinoma

IBD – inflammatory bowel disease

Ltx – liver transplantation

MR – magnetic resonance

PET – positron emission tomography

PSC – primary sclerosing cholangitis

UC – ulcerative colitis

US – ultrasound

This thesis is based on the following papers referred to by their Roman numerals:

- I.** Bergquist A, Glaumann H, Persson B, Broomé U. Risk factors and clinical presentation of hepatobiliary carcinoma in patients with primary sclerosing cholangitis – a case control study. *Hepatology* 1998;27:311–316.
- II.** Fleming K A, Boberg K M, Glaumann H, Bergquist A, Smith D, Clausen O P. Biliary dysplasia as a marker of cholangiocarcinoma in primary sclerosing cholangitis. *Journal of Hepatology* 2001;34:360–365
- III.** Bergquist A, Glaumann H, Stål P, Wang G-S, Broomé U. Biliary dysplasia, cell proliferation and nuclear DNA fragmentation in primary sclerosing cholangitis with and without cholangiocarcinoma. *Journal of Internal Medicine* 2001;249:69–75.
- IV.** Bergquist A, Tribukait B, Glaumann H, Broomé U. Can DNA cytometry be used for evaluation of malignancy and premalignancy in bile duct strictures in primary sclerosing cholangitis? *Journal of Hepatology* 2000;33:873–877.
- V.** Bergquist A, Ekbohm A, Olsson R, Kornfelt D, Lööf L, Danielsson Å, Hultcrantz R, Lindgren S, Prytz H, Sandberg-Gertzén H, Almer S, Granath F, Broomé U. Hepatic and extrahepatic malignancies in primary sclerosing cholangitis. Submitted to *Journal of Hepatology*.

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BACKGROUND

Primary sclerosing cholangitis (PSC) is a chronic progressive cholestatic liver disease of unknown causes, although immunological factors have been suggested to be of pathogenetic importance. The disease is strongly associated with inflammatory bowel disease (IBD). The first published case of PSC was presented by Miller in 1927 (1). Throughout the following 50 odd years, the disease remained a medical rarity, and by 1980 only about 100 cases had been published in the English literature (2). With the increased use of endoscopic retrograde cholangiopancreatography (ERCP) and clinical awareness of the relationship between PSC and IBD, more cases are identified. The true prevalence of PSC is not known, but in Sweden, the prevalence of PSC can be estimated to be 6 per 100,000 (the prevalence of ulcerative colitis (UC) is 170 per 100,000 inhabitants, and 3.7% of the patients with UC also have PSC) (3). In Norway, the point prevalence shows similar figures (8.5 PSC patients per 100,000 inhabitants) (4). The prevalence in the southern part of Europe, however, seems to be lower, as indicated by a Spanish study (only 2.2 PSC patients per million inhabitants) (5).

Diagnosis of PSC

The diagnosis of PSC is based on a combination of clinical, biochemical, histological and – most importantly – cholangiographic features, with irregularities and beadings of the intrahepatic and/or extrahepatic bile ducts (Figure 1) (6, 7). Secondary causes of sclerosing cholangitis, such as previous biliary tract surgery, biliary stone disease, congenital biliary tree abnormalities, cholangiopathy associated with AIDS, or bile duct neoplasm must be excluded (8). The classic laboratory finding in patients with PSC is an increased alkaline phosphatase serum level (7, 9, 10); however approximately 10% of the patients will present with normal alkaline phosphatase levels. Characteristic features in liver biopsies from patients with PSC

are: bile duct proliferation, periportal inflammation and fibrosis, and obliteration – and finally loss – of bile ducts (11). The extent of fibrosis is often scored into four stages (I–IV), where stage IV represents cirrhosis. It is, however, important to be aware of the considerable sampling variability in liver biopsies (12).



Figure 1. A cholangiogram from a patient with known PSC and cholangiocarcinoma. As shown, there are irregularities in the biliary tree, making identification or exclusion of a cholangiocarcinoma difficult.

Natural history of PSC

The mean age at PSC diagnosis is 32 to 42 years; about two – thirds of the patients are men (5, 7, 10, 13). PSC is also seen in children but is less frequent than in adults. The clinical presentation of PSC can vary greatly, and the patients may either be symptomatic or asymptomatic. In the symptomatic group, the disease is characterised by periodic exacerbations and remissions, the most common symptoms being fatigue, jaundice, pruritus, and abdominal pain (7, 10). 15 – 45% of the patients are asymptomatic at the time of diagnosis and may remain so despite progression of the disease (6, 10, 13). 22–53% of the initially asymptomatic patients develop symptoms during a follow-up period of 6 years (10–14). PSC frequently progresses to cirrhosis, and complications – such as fever, bacterial cholangitis, biliary stones, abdominal pain, dominant biliary strictures, hepatic failure, portal hypertension and cholangiocarcinoma – are common. Since at the present time there is no treatment available to effectively halt the progression of PSC many of the patients with end stage cirrhosis need liver transplantation – a surgical intervention having a 1-year survival of almost 90% (15). The median survival time from diagnosis to death, or liver transplantation, is reported to be around 12 years (6, 10, 16).

Associated diseases

PSC has been found to be strongly associated with IBD, most commonly UC. The prevalence of UC in patients with PSC varies between 70 and 100% (9, 10, 17). Most UC patients having PSC suffer from pancolitis, the prevalence of PSC being 5.5% in patients with pancolitis and 0.5% in patients with distal colitis (3). The colitis in PSC is characterised by a quiescent course and a higher risk for colorectal cancer/dysplasia than in UC patients without PSC (18–22). The risk of developing colorectal cancer in PSC patients with UC is further discussed on

page 19. The second most common disorder associated with PSC appears to be pancreatitis. 15–46% of all PSC patients have radiological changes indicative of chronic pancreatitis (23–25). However, the pancreatic changes seen in PSC are mild, and clinically important exocrine failure is rare (24). Moreover, other autoimmune disorders, for example celiac disease and diabetes mellitus, are more frequent in patients with PSC than in IBD patients without PSC (26).

Cholangiocarcinoma in PSC

An association between UC and cholangiocarcinoma was first described by Parker and Kendall in 1954 (27). Later, in 1971, Converse et al found that bile duct carcinoma in UC most commonly occurs in patients with pre-existing PSC (28). Today, we know that PSC can be complicated by cholangiocarcinoma, as well as gallbladder and hepatocellular carcinoma (HCC) (29). The increased risk for developing cholangiocarcinoma in PSC is well established, the prevalence varying in different studies between 8 and 20% (10, 30–32). The main reasons for the variation in the number of PSC patients found to have cholangiocarcinoma are probably differences in selection of patients, diagnostic ambitions, and autopsy rates. This is well reflected by data from a Swedish study including 305 PSC patients (10). The overall prevalence of cholangiocarcinoma in this study was 8%. However, among PSC patients with a follow-up of more than 5 years, 16% developed cholangiocarcinoma. 79 (26%) patients in this study died or underwent liver transplantation, and 30% among them were found to have cholangiocarcinoma. In the group of PSC patients who died, 69% were autopsied. Results of several studies show that cholangiocarcinoma in some PSC patients will only be revealed by autopsy (33). Moreover, in 30–50% of all PSC patients with hepatobiliary malignancy, cholangiocarcinoma is diagnosed concomitantly with PSC (34). Some of these patients may not have an underlying PSC, since cholangiocarcinoma without PSC may present with

cholangiographic changes similar to those seen in PSC patients without cholangiocarcinoma. The tumour can be located intra – or extrahepatically. In a study by Ahrendt et al including 25 PSC patients with biliary malignancy, 76% of the tumours were located in the perihilar region, 16% were intra hepatic and 8% were located in the gallbladder (34). Cholangiocarcinoma in PSC typically develops when the patients are in their 40's, i.e., about 20 years earlier than cholangiocarcinoma in patients without PSC (30, 35). The prognosis is dismal, with a medium survival of 5 months after the cholangiocarcinoma diagnosis is established (30).

Pathogenesis of cholangiocarcinoma in PSC

Cholangiocarcinoma is a malignant proliferation of bile duct epithelial cells that can arise anywhere in the biliary tree. The factors responsible for the malignant transformation of the bile duct epithelium in PSC are not known. Cholangiocarcinoma in PSC patients can arise at any stage of the disease (36). In a recent report by Ahrendt et al, only 20% of cholangiocarcinoma patients had concomitant cirrhosis (34). PSC is characterised by proliferation of the bile ducts and periductal fibrosis caused by a chronic inflammation. The fact that patients with chronic clonorchis sinensis and opisthorchis viverrini (liver fluke) infection also run an increased risk of developing cholangiocarcinoma suggests that any chronic inflammation of the bile ducts enhances the risk for malignancy. When the cholangiocytes are continuously exposed to inflammatory agents and hydrophobic bile acids, the cells may become predisposed to oncogenic mutations and further progression to the malignant state. The role of activated onc-genes and functional loss of tumour suppressor genes in the pathogenesis of cholangiocarcinoma in PSC is enigmatic. K-ras mutations was found in 33% of cholangiocarcinomas from patients with PSC (37, 38); similar figures were reported concerning cholangiocarcinoma without PSC (39–41). Mutation of p53 in PSC-related cholangiocarcinoma is reported to vary between 30 and 80% (37, 38, 42). In addition,

loss of chromosome 9p21, and inactivation of the p16 tumour suppressor gene – both with critical roles in the cell cycle machinery – have been shown to be common events in PSC associated cholangiocarcinoma (43) as well as in cholangiocarcinoma without PSC (44). Failure to activate apoptosis and to dispose of cells with genetic damages are additional possible mechanisms by which malignancy can arise. The balance between proliferation and apoptosis plays an important role in tissue homeostasis. In biliary diseases, excessive apoptosis due to, for example, immunomediated processes or toxic agents (i.e. biliary acids) leads to ductopenia, and – inversely – inhibition of apoptosis may lead to bile duct proliferation and possibly development of cholangiocarcinoma (45). The significance of tissue homeostasis for the balance between apoptosis and cell proliferation is schematically illustrated in Figure 2. The role of apoptosis in the carcinogenesis of PSC remains, however, obscure. Usually, a genetically damaged cell is either repaired or subjected to apoptosis. If apoptosis is impaired, the genetic damage may become fixed and cancer can develop through a multi-step process. An illustration of the possible role of apoptosis for the development cholangiocarcinoma in PSC is given in Figure 3.

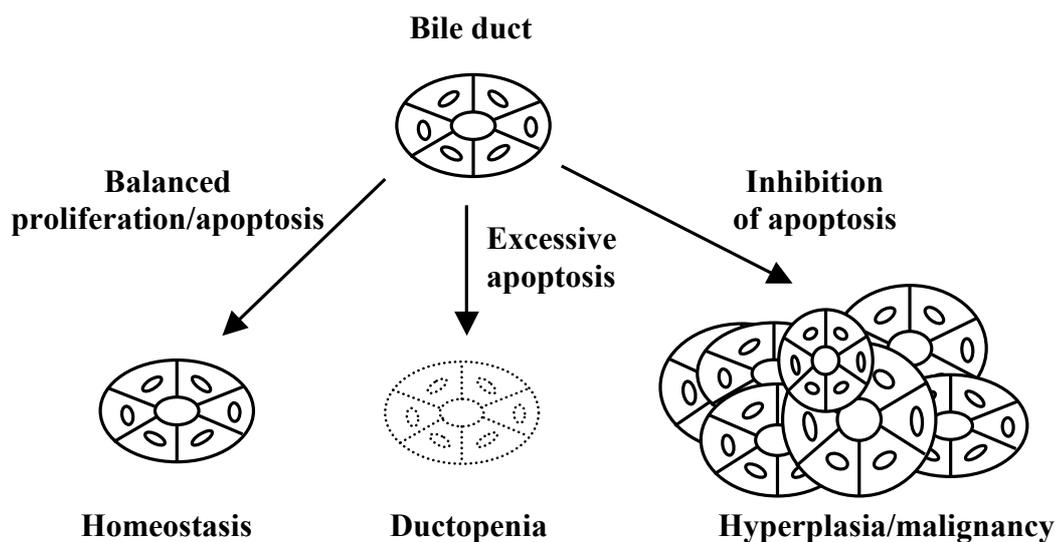


Figure 2. The balance between apoptosis and cell proliferation is important for tissue homeostasis. If apoptosis is inhibited, an opportunity for hyperplasia and malignancy is provided. Excessive apoptosis leads to increased cell death and ductopenia (45).

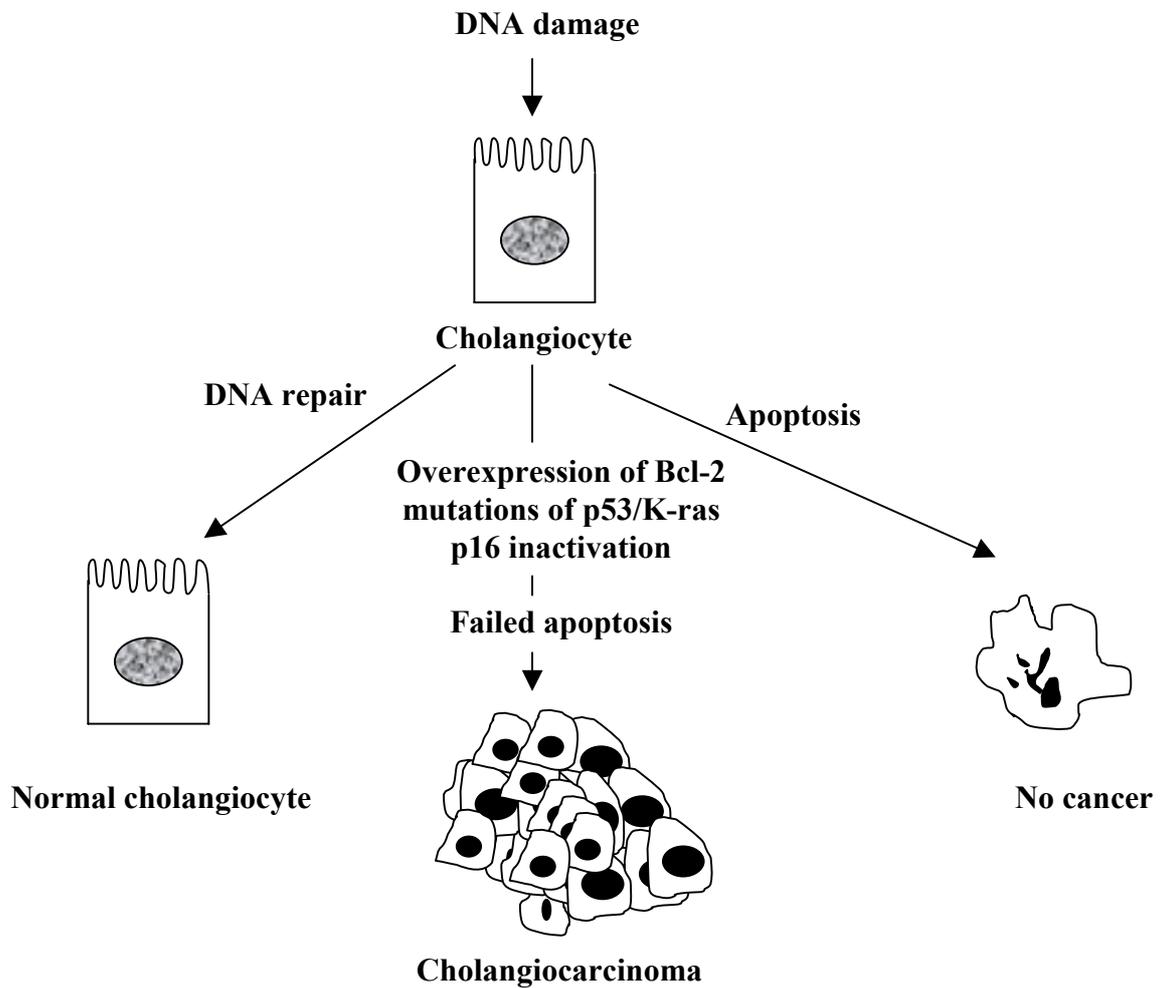


Figure 3. Apoptosis is possibly important for the development of cholangiocarcinoma in PSC. If apoptosis is impaired, the genetic damage may become fixed. Effective apoptosis removes cells with serious genetic damage—beyond repair (45).

Several cancers present pre-malignant changes (dysplasia) prior to true malignancy. In the biliary tract, bile duct dysplasia may be a morphological step in the transition from benign to malignant bile duct epithelium. Biliary dysplasia has been reported in a few cases of PSC to precede the development of cholangiocarcinoma by up to 18 months (46). The concept of biliary dysplasia has been controversial and, in a study by Ludwig et al, biliary dysplasia was found only in one out of 60 PSC patients who underwent liver transplantation, but none of these patients had cholangiocarcinoma (47).

Diagnosis of cholangiocarcinoma in PSC

Clinical signs and symptoms

Cholangiocarcinoma in the setting of PSC is difficult to reveal, and is often diagnosed at an advanced stage of tumour growth and spread, or incidentally at liver transplantation in end stage PSC (30, 48–50). Clinically, biliary malignancy is often suspected when a PSC patient shows signs of rapid, progressive liver disease with increasing bilirubin levels, weight loss and abdominal pain. In a study by Nashan et al (49), the old Mayo Model risk score – based on a formula including bilirubin level, histological stage, age, and presence of splenomegaly (51) – was evaluated in 48 PSC patients undergoing liver transplantation. 10 patients suffered from cholangiocarcinoma, the cancer being incidentally found in 9 of these patients. A marked increase in the incidence of biliary malignancy was shown at a Mayo Model risk score above 4.4. However, one year before transplantation, patients with biliary malignancy did not differ from non-cancer patients in their clinical course. Therefore, Mayo Model scoring may be helpful in evaluating the probability of cholangiocarcinoma in PSC patients immediately prior to liver transplantation.

Moreover, it is difficult to differentiate between malignant and non-neoplastic strictures cholangiographically, since fibrotic strictures of the bile ducts are present already at the onset of the disease process in PSC. Both ultrasound (US) and computer tomography (CT) have a low sensitivity for the detection of cholangiocarcinoma in PSC (7% and 29%, respectively) (52). In a retrospective study, by Campbell et al encompassing PSC patients with cholangiocarcinoma, the benefit of CT, cholangiography, US and magnetic resonance (MR) imaging in demonstrating cholangiocarcinoma was evaluated (53). The most sensitive method was MR imaging, although this was only restricted to a minority of the patients. Cholangiocarcinoma could be detected in 80% of the patients using a combination of various radiological methods. However, only PSC patients with cholangiocarcinoma were included in the study (53). MR imaging is a non-invasive diagnostic tool with good accuracy for diagnosing PSC (54). However, the feasibility of this method for detecting cholangiocarcinoma in PSC needs to be further evaluated. Positron emission tomography (PET) using a radiolabelled glucose analogue accumulating in malignancies was evaluated as a tool for detecting cholangiocarcinoma in PSC, in a report from Keiding et al (55). This study shows promising results regarding the possibility of revealing small cholangiocarcinomas in PSC, although the applicability of the method is limited by its low availability.

Histology and brush cytology

Bile duct carcinomas in PSC often show a desmoplastic (scirrhous) growth pattern; even if a change suspected of being malignant is found by CT or US, it may therefore be difficult to obtain a representative biopsy specimen. Brush cytology from strictures obtained at ERCP has a good specificity, but a relatively low sensitivity and the method is therefore of limited value in diagnosing cholangiocarcinoma in PSC (56–59). Repeated brushings can increase the sensitivity (58) and are recommended when there is a strong suspicion of cholangiocarcinoma

and the initial material is negative or non-conclusive. Despite the high specificity, false positive cases occur. Ponsioen et al (59) have recently evaluated the efficacy of brush cytology in dominant PSC strictures. The study included 47 brush samples from 43 PSC patients; sensitivity and specificity for cholangiocarcinoma diagnosis were 60% and 89%, respectively. Immunohistochemical analyses of p53 and K-ras mutations were also made but did not supply additional evidence for the diagnosis of malignancy. However, in a recent study including 117 patients with biliary strictures in patients without PSC, detection of K-ras mutations in bile specimens improved the diagnosis of malignancy over histology alone (60). It is difficult with these methods to differentiate between reactive cellular atypia generated by chronic inflammation, and true neoplasia. DNA measurements by image cytometry on cytology specimens from strictures in PSC and cholangiocarcinoma is another means of detecting malignant transformation of bile duct epithelium and will increase the sensitivity over cytologic analysis only (61, 62).

Tumour markers

The carcinoembryonic antigen (CEA) and the carbohydrate antigen 19-9 (CA 19-9) are oncofetal antigens found in high concentrations in gastrointestinal carcinomas. Rising titres of the tumour markers CEA and CA 19-9 in the blood support a suspicion of cholangiocarcinoma. In a study published in 1995 by Ramage et al, the use of a combination of the two tumour markers CA 19-9 and CEA was evaluated. The results showed an 86% accuracy in diagnosing cholangiocarcinoma when the formula $(CA\ 19-9 + (CEA \times 40)) > 400$ was applied (52). In the quoted study, eight patients had incidental cholangiocarcinoma diagnosed after transplantation. Two of the eight patients had a tumour marker score of < 400 and were the only patients in this group with a survival without tumour recurrence of more than 6 months after liver transplantation, indicating that tumour markers are of limited benefit

for the early detection of cholangiocarcinoma in PSC. This notion is supported by the findings in a study by Hultcrantz et al, in which an effort to diagnose cholangiocarcinoma in an early stage was made: Four tumour markers (CA 19-9, CEA, CA 50, CA 242) were evaluated in 75 patients prospectively observed for 3 years (36). Two patients developed cholangiocarcinoma, while one had normal and one increased serum tumour marker levels. Transient, non-cholangiocarcinoma associated elevations were seen in five patients. During follow-up (8 yrs.), two additional patients developed cholangiocarcinoma, none of whom had earlier shown raised tumour markers. It was concluded that these tumour markers lacked both in sensitivity and specificity (36). In another study, repeated measurements of CA 19-9 and CEA were made in 36 PSC patients without cholangiocarcinoma (63). Among these patients, nearly 40% had increased levels of CA 19-9 at some occasion. The Ramage score was applied on this group and was found to have a low sensitivity (33%) but a high specificity (85%). In summary, CEA and CA 19-9 have a role as diagnostic tools in the revelation of cholangiocarcinoma in patients with PSC. The Ramage formula is recommended for use, but awareness of the risk of false negative as well as false positive results is important. Measurements of tumour markers in the bile have given contradictory results. In one study, biliary CA 19-9 was very sensitive and specific in detecting malignancy (64). However, in a more recent study by Björnsson et al, analyses of CEA and CA 19-9 in the bile were not found to have any advantages over serum analyses (63). A summary of the sensitivity and specificity of various tools for diagnosing cholangiocarcinoma in PSC is presented in Table 1.

Table 1. Diagnostic tools for detecting cholangiocarcinoma in PSC

Method	Sensitivity	Specificity
Mayo Model Risk Score (49)	Moderate	Moderate
Ultrasound/computer tomography/ERCP (52, 53)	Poor	Poor
Tumour markers (CA 19-9, CEA) (36, 52, 63)	Moderate	Moderate
PET (55)	Excellent(?)*	Excellent(?)*
Brush cytology (57, 59)	Poor	Excellent

* needs to be confirmed

PSC patients at increased risk for developing cholangiocarcinoma

Cholangiocarcinoma is a leading cause of death in PSC and the poor prognosis after liver transplantation for cholangiocarcinoma necessitates the identification of PSC patients at risk for malignant development in order to make transplantation at an early stage. Tools for identifying PSC patients at increased risk for developing cholangiocarcinoma or pre-malignancy are lacking. Possible risk factors include presence of colorectal dysplasia/cancer in patients with concomitant UC (19), and alcohol consumption (32). Neither long duration nor complications or symptoms of PSC seem to be risk factors for cholangiocarcinoma in PSC (32, 49). In South East Asia, risk factors for cholangiocarcinoma to arise in patients without PSC include infestation with the parasites *clonorchis sinensis* (in Japan, Korea, and Vietnam) and *opisthorchis viverrini* (in Thailand, Laos, and Malaysia), in addition to smoking (65). K-ras mutations in bile fluid of PSC patients has also been suggested as a risk factor for later development of cholangiocarcinoma (66).

Treatment of cholangiocarcinoma in PSC

Liver transplantation is usually not recommended as treatment for cholangiocarcinoma since tumour recurrence is frequent. Results presented in a recent study, show that

cholangiocarcinomas diagnosed prior to liver transplantation have a poorer prognosis than incidentally diagnosed cholangiocarcinomas (67). Presence of incidentally detected cholangiocarcinomas less than 1 cm in diameter discovered at the time of gross examination of the explanted liver did not influence the patient's chances of survival (68). A combination of cytostatics and radiation has recently been presented from the Mayo Clinic for the treatment of cholangiocarcinoma in PSC with some promising results (69).

PSC and the risk of colorectal carcinoma

UC is a well known risk factor for the development of colorectal carcinoma. Long duration of the disease and extensive colitis are the two most important factors associated with this complication. Lately, a concomitant PSC has been shown to increase the risk of colorectal cancer or dysplasia in patients with UC (19–22). In a study from Sweden, the absolute cumulative risk of developing colorectal dysplasia/cancer in the PSC/UC group was calculated to be 9%, 31% and 50%, respectively, after 10, 20 and 25 years of disease duration. In the group with UC only, the corresponding risks were 2%, 5% and 10%, respectively (19). Almost identical cumulative incidence rates of colorectal neoplasia were found both in a recent case control study from Finland including 90 patients, and in a population based study from Sweden of 125 PSC patients (22, 70). Strangely, other studies lack evidence for the notion that PSC patients are at increased risk of developing colorectal dysplasia/cancer (71, 72). The dissent findings may be explained by differences in study designs. Patients with cholestatic liver disease have a decreased bile acid excretion and a relatively high proportion of secondary bile acids (73). It has been speculated that secondary bile acids play a role as carcinogenic agents in the colorectal mucosa. This theory is supported by the observation that right sided colorectal cancer seems to be more common in patients with PSC than in patients with UC alone (21, 74, 75). In a recent report by Tung et al, it was shown that treatment with

ursodeoxycholic acid decreased the risk for developing colorectal dysplasia in patients with PSC and UC. This study further supports the theory that secondary bile acids is a risk factor for the development of colorectal dysplasia/carcinoma in PSC (76). Since the colitis in PSC often runs a quiescent course (18), the presence and duration of UC in PSC may be underestimated. Colonoscopy with multiple biopsies should therefore be performed in PSC patients, and – if UC is found – colonoscopic cancer surveillance should be considered. PSC patients with UC remain at an increased risk of developing colon cancer/dysplasia even after they have undergone liver transplantation (77).

In summary, patients with PSC run an increased risk of developing colorectal cancer/dysplasia and cholangiocarcinoma. Tools for the early diagnosis of cholangiocarcinoma and possibilities to detect pre-malignancy are lacking. The general purpose of this thesis was to try to identify early diagnostic markers and risk factors for malignancy in PSC, as detailed in the following.

AIMS

The specific aims of the present study were

- to assess clinical features in patients with PSC and hepatobiliary carcinoma, and identify risk factors for the development of this cancer type (Papers I and V)
- to compare clinical, biochemical and radiological data in patients with PSC and hepatobiliary carcinoma with patients suffering from end stage chronic liver disease due to PSC (Paper I)
- to clarify the significance and morphologic expression of the concept of dysplasia of the biliary epithelium and the possibility of agreement on criteria for this concept; further to determine the reproducibility of the diagnosis of bile duct dysplasia using these criteria (Paper II)
- to compare the frequency of bile duct dysplasia in livers from patients with PSC with and without cholangiocarcinoma (Papers I, II and III)
- to study cell proliferation, apoptosis and expression of p53 and bcl-2 proteins in cholangiocarcinomas and in non-tumourous liver tissue from PSC patients with and without cholangiocarcinoma (Paper III)
- to evaluate DNA ploidy patterns in cholangiocarcinomas from patients with and without PSC (Paper IV)
- to identify a large cohort of Swedish PSC patients, and to assess the risk of developing hepatic and extrahepatic malignancies in this cohort compared to the risk in the general Swedish population (Paper V)

MATERIALS AND METHODS

Paper I

Patients

All patients with histologically proven PSC and cholangiocarcinoma, HCC or GBC treated at the Department of Gastroenterology at Huddinge University Hospital between 1984 and 1995 were included in the study (n=20). Every cancer patient was matched for age and sex to a control patient with PSC but without known cancer.

Data collection

All information was obtained by review of the complete medical history collected from patient files, and included the following data: (1) Family history of chronic liver or colonic disease and cancer; (2) concomitant autoimmune diseases; (3) information concerning smoking and intake of alcohol; (4) characteristics of PSC and IBD; (5) presence of symptoms such as abdominal pain, jaundice, itching, fever, ascites, weight loss, encefalopathy, fatigue; (6) laboratory data including serum electrophoresis, auto-antibodies, alpha-fetoprotein, CA 19-9 and markers for viral hepatitis; and (7) operative and medical treatment for PSC and IBD. Cholangiograms from 20 patients were re-evaluated according to a five-degree scale. Liver biopsies were re-reviewed to screen for foci of dysplasia in the bile ducts outside the tumour and to confirm the diagnosis of cancer. Smoking data was verified in all living patients by telephone interviews.

Statistical analysis

Mann-Whitney's U-test was used for comparison of continuous variables. For comparison of non-continuous variables, Chi square analyses or Fisher's exact test were used whenever applicable.

Paper II

Patients

The cases were selected from the files of the Nuffield Department of Pathology and Bacteriology, John Radcliffe Hospital, Oxford, UK; the Department of Pathology, National Hospital, Oslo, Norway; and the Department of Pathology, Huddinge University Hospital, Stockholm, Sweden. Two series of liver biopsies were examined: (1) Liver biopsies from 26 PSC patients with concomitant cholangiocarcinoma, or with development of cholangiocarcinoma in the subsequent 2 years; (2) liver biopsies from 60 patients with PSC (20 from the departments of pathology at each hospital), who did not develop cholangiocarcinoma in the subsequent 2–5 years (group 2).

Assessment of dysplasia

Group 1: The slides were randomly coded by a neutral observer and examined separately by the three pathologists. The pathologists were blinded as to the origin and history of the cases and scored the livers for dysplasia being “present”, “absent” or “possible”, using their own criteria (First Round). Thereafter, the pathologists reviewed the slides jointly at a multi-head microscope and agreed on criteria for dysplasia, shown in Table 2. The slides were randomly re-coded and re-scored six months later, by the pathologists separately, and in their own departments, using the agreed criteria (Second Round). Group 2: The 60 cases were assessed in the pathologist’s own departments using the same criteria as for the second round.

Comparison between the first and second rounds and between groups 1 and 2 were made. The undersigned author took active part in the evaluation of presence of bile duct dysplasia in round 2 (group 1 and group 2) together with one of the pathologists.

Statistical Analysis

Inter-observer agreement for the slides between the two rounds of scorings was evaluated using the κ coefficient of Cohen (78) as calculated by the computer package Stata (1997). The level κ ranges between 0 and 1, where 0 represents no agreement and 1 represents full agreement.

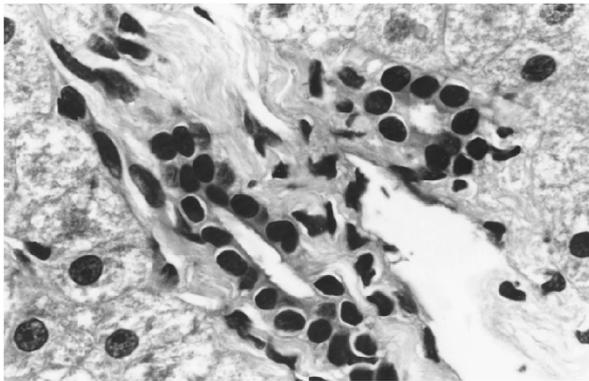


Figure 4. Normal interlobular bile ducts



Figure 5. Dysplastic bile duct

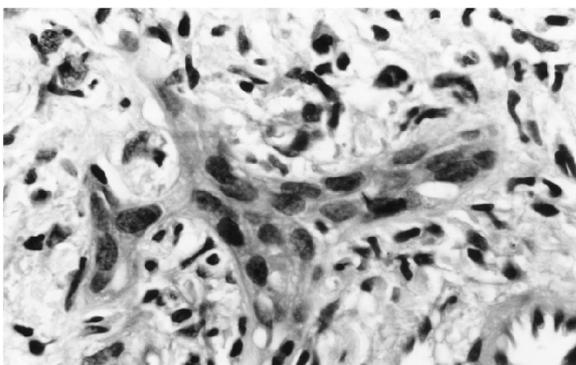


Figure 6. Hyperplastic bile duct

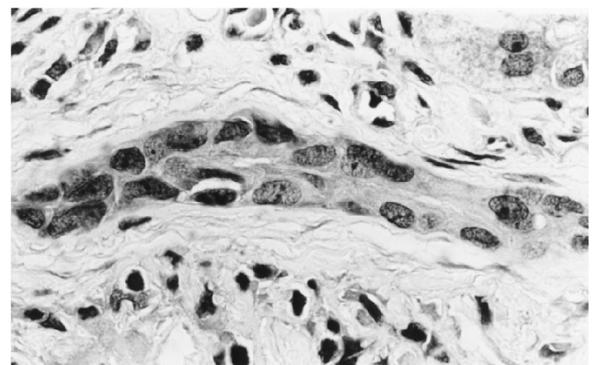


Figure 7. Changes borderline for dysplasia Interlobular bile duct

Table 2. Classification of normal and dysplastic biliary epithelium

If one duct is dysplastic, then the slide is scored as dysplastic.

Normal (Fig. 4)

Architecture of ducts

One or two interlobular ducts per portal tract, well-separated, epithelium is regular, one layered, cuboidal.

Cytology

Vesicular nucleus, round, regular, one or no nucleolus (inconspicuous)
Nuclear to cytoplasmic ratio (N:C) 1:2, no mitoses.

Dysplasia (Fig. 5)

Architecture of Ducts

Increased number of ducts, distension, larger than normal. Irregular shape, not round. More than one layer of epithelial cells, papillary infolding, cribriform.

Cytological

Nucleus enlarged stippled and hyperchromatic, varying shape and outline of nuclei, nucleoli increased and prominent, N:C 1:1.

Cells enlarged, mitotic figures.

Mucus production.

Degeneration/Regeneration (Fig. 6)

Architecture

Increased number of ducts, variable size and shape of ducts. May be some multi-layering of cells.

Cytological

Nuclei regular, vesicular, occasional mitoses. No hyperchromasia, N:C 1:2.

Nuclei shrunken with loss of nuclear and cytoplasmic detail.

Indefinite, Indeterminate, Borderline (Fig. 7)

Abnormalities which are between degeneration/regeneration and dysplasia.

Paper III

Patients

Liver and cancer tissue from 14 PSC patients with cholangiocarcinoma/GBC and liver tissue from 16 randomly selected patients with end-stage PSC without cancer were studied. 10 of the cancer patients and 15 of the controls were also included in Paper I.

Histology

Sections from non-cancerous areas of “benign” liver tissue were studied histologically by one hepatologist and one hepatopathologist and examined for foci of dysplasia and degree of bile duct proliferation. Bile duct dysplasia was diagnosed according to the criteria defined in Paper II. Expression of Ki-67, p53 and bcl-2 was ascertained immunohistochemically. Following antigen retrieval through microwave exposure, tissue sections were incubated with monoclonal antibodies, and a standard using the streptavidin-biotin method with 3,3'-diaminobenzidine as the chromogen was used. The ApopTag® in situ detection Kit (Oncor Inc., Gaithersburg, Md, U.S.A.) was used to demonstrate nuclear DNA-fragmentation by immunoperoxidase detection of digoxigenin-labelled genomic DNA in formaldehyde-fixed liver sections. Areas on the slides were randomly selected for analysis, and in each case more than 1000 bile duct or tumour cells were counted. The number of stained cells were expressed as the labelling index (LI): the number of stained nuclei in percent of the total number of nuclei.

Statistical analysis

Mann-Whitney's U-test was used for comparison of continuous variables. For comparison of non-continuous variables, Chi-square analyses, or Fisher's exact test were used whenever applicable.

Paper IV

Patients

28 cases of cholangiocarcinoma were available for DNA analysis; they were identified from a register including all cancer cases diagnosed at Huddinge University Hospital between 1989 and 1997 and were all included in the present study. 10 of the patients had PSC. 17 samples from large or middle sized bile ducts from 15 transplanted PSC patients without cholangiocarcinoma were also analysed. 22 of the PSC patients were also included in Paper III. Benign gallbladder tissue consecutively obtained from 100 patients without PSC but with chronic cholecystitis was used as an additional control group (79).

Histology and flow cytometry

All slides from tumours were re-examined by one hepatologist and one hepatopathologist and graded histologically. All slides from non-cancerous PSC patients were also re-evaluated in order to exclude signs of malignancy or biliary dysplasia/atypia. A sample approximately 3x3 mm of the tumour (or bile duct/gallbladder) tissue was cut from the block after marking the area of interest on an adjacent slide. After deparaffinizing with xylene and rehydrating by ethanol and water, the biopsies were incubated with 200 µL subtilisin Carlsberg solution (0,1% Sigma protease XXIV, 0,1 M Tris, 0,07 M NaCl, pH 7,2) in order to disintegrate the selected specimen. Staining was accomplished out by directly adding 1 ml DAPI (4',6-diamidine-2'-phenylindole-dihydrochloride)-phosphate solution (10 µM DAPI, 800 mM disodium-hydrogenphosphate) to the 1 ml subtilisin Carlsberg solution (80). The stained nuclei were analysed using a PAS II flow cytometer (Partec, Münster, Germany) equipped with a 100 W mercury lamp. DAPI was excited at 365 nm and the fluorescence was measured above 435 nm. Samples with a single peak were regarded as diploid while those with an additional peak were regarded as aneuploid. The multicycle program for cell cycle analysis

established by P.S. Rabinovitch (Phoenix Flow Systems, San Diego, Ca) was used for histogram analysis. The sliced nuclei option of this program for subtraction of the background combined with the correction of clumping was used for calculations of percentages of nuclei in S- and G2-phases.

Statistics

Comparisons of clinical variables in the different groups was made using the Chi-square test or Mann-Whitney's U-test for continuous variables. The Kaplan-Meier method for survival was analysed using log rank test.

Paper V

Patients

The study cohort comprised all PSC patients on file in all university hospitals in Sweden between 1970 and 1998. 604 patients with an ERCP-verified diagnosis of PSC were identified by members of the Swedish Internal Medicine Liver Club, representing all Swedish university hospitals. All patients' records were scrutinised according to a protocol, agreed upon by all participants, to confirm the diagnosis of PSC and to register presence of IBD and colorectal dysplasia, colectomy, autopsy and diagnosis of malignancy.

Follow-up

Two strategies were used for follow-up: 1) Through the National Swedish Cancer Registry which provided data on all cancer patients until Dec 31, 1996; and 2) through the hospital records (latest clinical visit), no later than June 30, 1998. From the Cause of Death Registry, we ascertained date of death during follow-up and also checked if any cause of death due to cancer was reported that was not present in the files of the National Swedish Cancer Registry.

Endpoints were death, or last date of clinical follow-up. Patients were also censored at time for liver transplantation.

Statistical method

Comparison of non-continuous variables was made using Chi square analysis or Fisher's exact test whenever appropriate. For comparison of continuous variables Student's *t*-test was used. The Kaplan-Meier method was used to calculate life table estimates for contracting and for dying after diagnosis of hepatobiliary carcinoma. The expected number of cases used to calculate SIRs were obtained by multiplying age and calendar specific person-years in the cohort with the corresponding incidence in the entire Swedish population. Confidence intervals of SIRs were calculated assuming Poisson distributed number of observed cancer cases. Cox' regression analysis was applied to evaluate the risk of developing hepatobiliary carcinoma in PSC patients with concomitant colitis.

All studies were approved by the local ethics committee.

RESULTS

Paper I

Clinical data

Mean age in the cancer group (n=20) was 45 (\pm 10) years and in the control group (n=20) 44 (\pm 11) years. The mean duration of PSC 10 (\pm 7) years vs. 12 (\pm 7) years did not differ between the cancer and control groups. There were no significant differences in the duration of IBD, IBD activity, number of patients on whom colectomy had been performed, or the presence of colonic dysplasia/cancer between cancer patients and controls. The only symptom that differed between cancer and non-cancer patients was the presence of abdominal pain localized in the upper right quadrant at time of endpoint ($p < 0.05$). Laboratory data including alkaline phosphatase, serum transaminases, prothrombin and albumin did not differ in comparison between cancer patients and controls. Measurement of CA 19-9 was available in ten cancer patients and seven controls. The mean level of CA 19-9 in the cancer group (n=10) was 700 kU/l and in the control group (n=7) 46 kU/l ($p < 0.05$).

Smoking and alcohol

The number of patients being either current or former smokers was significantly higher in the cancer group than among the controls ($p < 0.0004$) as revealed in Table 3. To validate smoking data, the undersigned author personally re-interviewed all patients included in the study, who were still alive, and no changes in smoking data were disclosed.

Table 3. Smoking habits in 20 patients with PSC and liver cancer compared with 20 control patients with end stage PSC

Smoking habits	PSC patients with cancer in the liver (n=20)	PSC patients without cancer in the liver (n=20)	
Current smoker	4 (20%)	0	p<0.0004
Former smoker	6 (30%)	0	
Never smoker	10 (50%)	20 (100%)	

Histology and radiology

17 patients had cholangiocarcinoma, two HCC and one gallbladder carcinoma. 10 (50%) of the cancer patients and 18 (90%) of the controls had cirrhosis. Information about cirrhosis was not available in five cancer patients. The cholangiograms showed changes due to PSC with multiple and severe stenoses in both intra-and extrahepatic bile ducts in 85% (17/20) of the re-evaluated PSC patients. One of the seven patients with malignant disease had cholangiographic changes suspicious of cholangiocarcinoma. Among the PSC patients without cancer, three patients with strictures suspicious of malignancy were found. None of the registered cholangiographic parameters showed significant statistical differences between patients with benign and malignant disease.

Paper II

Group 1 – Patients with co-existing or later developed cholangiocarcinoma

The reproducibility in the first round for all three categories was extremely poor, the best figure being the one for dysplasia ($\kappa = 0.1293$ (slight agreement)). The results for absent or possible dysplasia were less than zero, indicating no better than random association. In contrast, the second round showed marked improvement in agreement, the kappa figure for dysplasia being 0.44 (moderate agreement). In biopsies where all three pathologists agreed, dysplasia was present in 23% (6/26) and 19% (5/26) of cases for round 1 and 2, respectively.

The three pathologists agreed that dysplasia was not present in 4% (1/26) and 31% (8/26, including one indeterminate case) of biopsies for rounds 1 and 2, respectively. In the remaining 73% (19/26) of cases in round 1 and 50% (13/26) of cases in round 2, none of the three pathologists agreed.

Group 2 –Cases without cholangiocarcinoma

Dysplasia was not found in any of these cases. Comparison of the incidence of dysplasia in this group (0%) with group 1 (19%) using Fisher's exact test showed that the difference was highly significant ($p < 0.001$).

Paper III

Tumourous tissue

Samples from 14 cholangiocarcinomas and 2 GBCs from patients with PSC were evaluated. Four tumours (25%) were negative for the p53 protein, while in three tumours more than 50% of the cells expressed p53. The number of Ki-67 protein positive bile duct cells in the tumour cells exceeded the number of cells staining positively for nuclear DNA-fragmentation by a factor of four, 22.8% vs. 5.6% ($p < 0.01$). Significantly higher levels of nuclear DNA-fragmentation and levels of Ki-67 and p53 proteins were noted in the tumour tissue than in bile duct cells in non-tumorous liver tissue from PSC patients with and without cholangiocarcinoma. None of the tumours expressed the bcl-2 protein.

Non-tumorous liver tissue

Histological stage, presence of biliary dysplasia and immunohistochemical staining for Ki-67, nuclear DNA fragmentation, p53 and bcl-2 in non-tumorous liver tissue from PSC patients with and without CC are shown in Table 4. Patients with bile duct dysplasia (n=9) had a

significantly higher frequency of moderate/marked bile duct proliferation than those without biliary dysplasia ($P < 0.01$).

Table 4. Histological characteristics and immunohistochemical staining for Ki-67 and nuclear DNA fragmentation in non-tumorous liver tissue from PSC patients with and without CC.

Histological characteristics	PSC patients with cholangiocarcinoma (n=12)	PSC patients without cholangiocarcinoma (n=16)	
No. of patients with stage III and IV fibrosis	12 (100%)	16 (100%)	NS
Bile duct dysplasia in non-tumorous liver tissue	7 (58%)	2 (13%)	$P < 0.05$
Moderate/marked bile duct proliferation	11 (92%)	7 (44%)	$P < 0.05$
Immunohistochemical staining	(n=12) mean LI in% (\pmSE)	(n=16) mean LI in% (\pmSE)	
Ki-67	1.1 (\pm 0.4)	0.6(\pm 0.1)	NS
Nuclear DNA fragmentation	1.0 (\pm 0.4)*	0.8 (\pm 0.2)	NS
p53 protein	0	0.02 (\pm 0.01)	NS
Bcl-2 protein	0	0.04 (\pm 0,03)	NS

* two samples from autopsies were excluded due to unspecific binding to all autolytic hepatocytes. LI=labelling index, SE = Standard Error

Paper IV

Patients without PSC (n=18) were significantly older than the PSC patients (n=10), with a mean age at cancer diagnosis of 67 years (\pm 14) and 47 years (\pm 15), respectively ($p < 0.01$). 54% (15/28) of all tumours showed non-tetraploid DNA aneuploidy. Tumours from patients with PSC displayed non-tetraploid DNA aneuploidy significantly more often (80%) than tumours

from patients without PSC (39%) ($p < 0.05$). There was no correlation between degree of histological differentiation of the tumours and DNA ploidy.

In PSC patients without cancer, 12% (2/17) of the samples displayed DNA aneuploidy. A comparison of DNA aneuploidy in benign bile ducts and cholangiocarcinoma in patients with and without PSC is shown in Table 5. Analyses from the 100 benign gallbladder samples revealed one case with DNA aneuploidy (1%) (79).

Table 5. Comparison of DNA aneuploidy in benign bile ducts and cholangiocarcinoma in patients with and without PSC

	Patients with DNA-aneuploidy	P-value
Benign bile ducts versus all CC (PSC and non-PSC)	12% (2/17) vs. 54% (15/28)	<0.005
Benign bile ducts versus CC in PSC	12% (2/17) Vs 80% (8/10)	0.0007

Paper V

Patient characteristics

604 patients with an ERCP-verified diagnosis of PSC were identified and total follow-up time was 4123 person-years. Median time of follow-up to end point was 5.7 (0-27.8) years (range). 15% (92/604) of the patients were treated with liver transplantation. 28% (171/604) of the patients were dead at last date of follow-up. The cause of death was cancer-related in 44%, liver related in 37% and due to other causes in 19%. The autopsy frequency among all deceased patients was 39% (66/171).

Risk of cancer

167 cases of cancer (including 18 colorectal carcinomas in situ) were identified in the cohort of the 604 patients. 112 patients had one cancer, 26 patients had two primaries and 1 patient had 3 different primary cancers. 21 of the cancer cases were diagnosed after Dec. 31, 1996, and could therefore not be identified through the National Swedish Cancer Registry. The frequency of hepatobiliary malignancies among the 604 PSC patients was 13.3% (81/604). The standardized incidence ratio (SIR) for hepatobiliary carcinoma was 161, as shown in Table 6. 37% (30/81) of all hepatobiliary malignancies were diagnosed less than one year after the diagnosis of PSC. The incidence rate was 1.5% per year, when patients diagnosed within a year after PSC diagnosis were excluded. The fraction of cancer free survival in the whole cohort is shown in Figure 4. The risk of hepatobiliary carcinoma in PSC patients and concomitant colitis with a history of colorectal carcinoma/dysplasia leading to colectomy was not higher than among patients without colorectal cancer/dysplasia. Colorectal cancer was only observed in patients with concomitant IBD, with a cumulative incidence of carcinoma of 7.4% (35/476). 23 (4.8%) patients with IBD had prevalent colorectal carcinoma or carcinoma in situ at the time of PSC diagnosis. 13 patients had both hepatobiliary malignancy and colorectal carcinoma/dysplasia. SIRs for colorectal carcinoma and for all gastrointestinal cancers are shown in Table 4. The PSC patients had a 10–14 times higher risk of developing pancreatic carcinoma than the general population. The overall cancer risk, when hepatobiliary cancers and colorectal carcinoma were excluded was not increased.

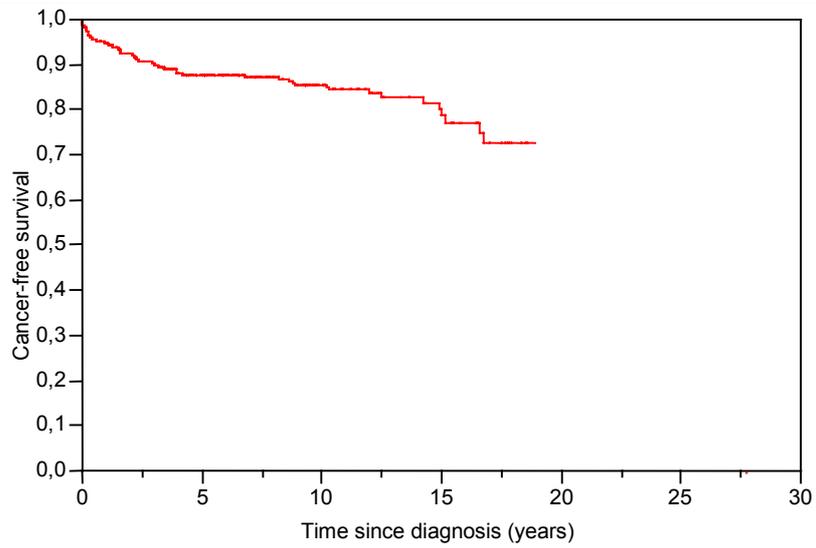


Figure 4. Fraction of surviving patients without hepatobiliary cancer over time in 604 patients with primary sclerosing cholangitis.

Table 6. Standardized incidence ratios (SIRs) for first cancer after diagnosis of primary sclerosing cholangitis, including and excluding first year after PSC diagnosis.

Site of cancer	Obs	Exp	SIR	95% CI	Excluding
all sites	87	14.3	6.1	(4.9–7.5)	–
all sites excluding colon – rectum and liver	16	11.8	1.4	(0.8–2.2)	–
gastrointestinal tract	71	2.5	28.6	(22.4–36.1)	–
oesophagus	0	0.1	0.0	(0–30.5)	–
stomach	1	0.4	2.2	(0.1–12.5)	–
small intestine	0	0.1	0.0	(0–50.5)	–
colon – rectum	12	1.2	10.3	(5.3–18.1)	–
liver*	53	0.3	161	(120–210)	–
pancreas	5	0.3	14.3	(4.7–33.4)	–
oesophagus	0	0.1	0.0	(0–34.2)	1st year
stomach	1	0.4	2.5	(0.1–14.1)	1st year
small intestine	0	0.1	0.0	(0–56.8)	1st year
colon – rectum	7	1.0	6.8	(2.7–14.0)	1st year
liver*	31	0.3	107	(72.6–152)	1st year
pancreas	3	0.3	9.7	(2.0–28.4)	1st year

* includes hepatocellular and bile duct carcinoma

GENERAL DISCUSSION

Patients with PSC run an increased risk of developing cholangiocarcinoma and the prognosis for patients with PSC and cholangiocarcinoma is dismal. Diagnostic tools for early cancer diagnosis and marker for pre-malignancy as well as risk factors for cholangiocarcinoma are lacking. The purpose of this thesis was therefore to study malignancy in general in PSC and try to identify markers for early diagnosis and risk factors for malignancy, since transplanted PSC patients with early cancer may have a favourable prognosis (68).

In Paper I, smoking is suggested to be a risk factor for the development of cholangiocarcinoma in PSC. Smoking has previously been presented as a risk factor for bile duct and gallbladder cancer in patients without an underlying PSC (65, 81–83). Smoking has also been considered a risk factor for HCC both in HBsAg positive chronic carriers and for patients with primary biliary cirrhosis (84, 85). An inverse association between current smoking and the development of PSC in patients with UC has been presented by Loftus et al (86) and Van Erpecum et al (87). Their findings are in agreement with the results in Paper I where none of the PSC patients without cancer smoked. In a recent retrospective case-control study by Chalasani et al, a comparison between 26 PSC patients with cholangiocarcinoma and 87 patients with PSC – but without cholangiocarcinoma – was made to determine risk factors and possible predictors for cholangiocarcinoma in PSC. In their study smoking was not found to be a risk factor for cholangiocarcinoma. However, the interpretation of the findings in the study is hampered by a possible selection bias since the patients were collected from eight different academic centers throughout the United States. In addition, smoking data were not confirmed by follow-up, as was the case in our single-center study, in which furthermore, all smoking data had been assessed by the same investigator in all cases.

The findings in Paper I also illustrate extreme difficulty in distinguishing PSC patients with end stage disease from those with liver malignancy, judging from the clinical and biochemical presentation. Only meticulous sectioning of the liver – either at transplantation or at autopsy – disclosed the tumour in most cases in the study. The general opinion among clinicians holds that cancer patients show a rapid persistent deterioration with weight loss, pruritus and severe jaundice (48). However, as shown in Paper I, patients with end stage PSC liver disease also have a rapid deterioration due to liver failure. Rapid clinical deterioration should therefore not necessarily be interpreted as a sign of malignancy and should not disqualify a PSC patient from the chance of a life saving liver transplantation.

There are at least two important clinical objectives related to the high incidence of cholangiocarcinoma in PSC. First, the task of differentiating between neoplastic and benign bile duct strictures, and, second, to identify patients at increased risk for cholangiocarcinoma or with pre-malignant changes. It has been proposed that biliary dysplasia is a developmental stage in the neoplastic transformation of the biliary epithelium (88). However, the concept of biliary dysplasia has been controversial. In a study in 1992 by Ludwig et al (47), only one case of biliary dysplasia was found in 60 transplanted PSC patients without cholangiocarcinoma. The case report by Martins et al (46) two years later showed biliary dysplasia in two PSC patients prior to or simultaneously with the diagnosis of cholangiocarcinoma. The advantage of identifying PSC patients with pre-malignant changes, such as biliary dysplasia, is obvious since these patients could be transplanted before malignancy occurs, thereby considerably improving the prognosis. In Paper II, an evaluation of the concept of biliary dysplasia has been made and criteria for biliary dysplasia are presented, with the aim of demonstrating their reproducibility. The results of the first round of analysis (where the pathologists used their own criteria, without prior discussion amongst themselves) show very poor reproducibility,

only slightly better than random. This is unacceptable for clinical purposes. However, none of the participating pathologists had previously routinely assessed dysplasia when evaluating liver biopsies from PSC patients. Reproducibility, after agreement among the participants on criteria around a multi-head microscope, was markedly improved in all categories of the classification system.

Problems in diagnosing biliary dysplasia are similar to those described in colorectal dysplasia include: inter-observer variations, high sampling variability (12), and difficulties in differentiating between biliary dysplasia and regeneration caused by chronic inflammation. The limitations in properly estimating biliary dysplasia may even be greater than in the case of colorectal dysplasia. In UC, several biopsies from the mucosa can easily be harvested at a colonoscopy. However, the risk of complications, such as bleeding and bile leakage, when taking liver biopsies, makes repeated sampling inappropriate, leading to a situation with less material for analysis. However, with an increased interest for the existence and significance of biliary dysplasia, the use and further evaluation of the criteria for this entity, better diagnostic accuracy will hopefully be achieved.

The presence of biliary dysplasia in 19% of cases with concomitant or subsequently developed cholangiocarcinoma (Paper II) indicates that dysplasia of ductal epithelium may be an early step in the pathogenesis of cholangiocarcinoma in PSC. This notion is supported by the results in Paper III, showing that patients with PSC and cholangiocarcinoma more often display bile duct dysplasia in non-tumorous liver tissue distant from the tumour (58%) than patients having end-stage PSC without cancer (13%). In addition, it is shown in Paper III that liver tissue with bile duct dysplasia significantly more often displayed moderate/marked bile duct proliferation than liver tissue without ductal dysplasia. Bile duct proliferation in patients with

cholangiocarcinoma could be secondary to infiltrative tumour growth and concomitant cholestasis. However, in this study, patients with end-stage cirrhosis had bilirubin levels similar to the ones in patients having PSC and cholangiocarcinoma. Furthermore, in Paper I, partly including the same patients, the cholangiograms in the two groups did not differ significantly. Bile duct dysplasia may sometimes be difficult to distinguish from reactive epithelial alterations; therefore, counting the number of bile ducts may be useful as a surrogate marker for premalignancy.

In Paper III, evaluations of cell proliferation, apoptosis, and the regulating factors p53 and bcl-2 were made. Non-tumorous liver tissue from PSC patients with cholangiocarcinoma was different from end-stage PSC without cancer in the expression of the p53 and bcl-2 protein or in nuclear DNA-fragmentation in bile duct cells. Therefore, at present, these markers are not useful for the early detection of cholangiocarcinoma in PSC. The malignant bile duct cells expressed significantly higher levels of Ki-67, the p53 protein and nuclear DNA-fragmentation than did bile duct cells in non-tumorous liver tissue from PSC patients with and without cholangiocarcinoma. This may have some clinical impact, since it is sometimes difficult to differentiate between benign and malignant bile duct cell proliferation in needle biopsies from patients with PSC. When diagnostic problems occur, high levels of Ki-67 and the p53 protein expression, and increased apoptosis (assessed as nuclear DNA-fragmentation) may conceivably serve as surrogate markers for cholangiocarcinoma in PSC.

Mutation of the p53 gene is one of the most common genetic events involved in human cancers (89, 90). Four tumours (25%) in Paper III were histologically negative for the p53 protein while in three tumours more than 50% of the cells were positive. This is less than in a previous report by Rizzi et al (42), where nearly 80% of PSC associated cholangiocarcinomas

expressed p53 in more than 80% of the tumour cells. However, in another investigation including 33 cholangiocarcinomas from patients with PSC, only 31% of the tumours expressed the p53 protein (37). In cholangiocarcinoma without PSC it is suggested that mutation of the p53 gene occur at a relatively late stage in the tumourigenesis (91). In the present study, neither areas with dysplastic bile duct cells nor non-dysplastic tissue show an overexpression of p53, indicating that the p53 mutation may be a late event also in the malignant transformation in PSC. However, wild type p53 expression is also detectable with immunohistochemistry, creating a risk of overestimation of p53 mutations when this method is used. In addition, we show that nuclear DNA-fragmentation was detectable in PSC associated cholangiocarcinoma. It was previously demonstrated that in situ detection of fragmented DNA fails to discriminate between apoptosis and necrosis (92). To counteract this bias in the present study, all histologically necrotic cells were excluded. It has been shown that liver tumour cells not only exhibit increased proliferation – there is also an increase in the number of cells undergoing apoptosis (93). The high rate of proliferating cells measured with Ki-67 labelling in the cholangiocarcinomas, and the lower rate of cells displaying nuclear DNA-fragmentation, indicate that dysregulated apoptosis is involved in tumour growth in PSC-associated cholangiocarcinoma. The almost total absence of bcl-2 expression indicates that downregulation of bcl-2 may be important for the dysregulation of apoptosis.

It is well established that dysplasia can be used as a marker of pre-malignancy in the colorectal mucosa in patients with longstanding UC (94), and colectomy is usually recommended in patients with high grade dysplasia. DNA aneuploidy correlates well with dysplasia in longstanding UC and may precede the appearance of dysplasia by several years (95, 96). Knowledge of the ploidy pattern of DNA in cholangiocarcinoma cells is a prerequisite for further studies on the possible correlation between biliary dysplasia and DNA

ploidy and DNA aneuploidy as a predictor for cholangiocarcinoma development in PSC. Therefore, the DNA content in cholangiocarcinomas was studied in Paper IV. 80% of the tumours from patients with PSC and cholangiocarcinoma showed DNA aneuploidy, and 12% of large bile duct epithelial linings from patients with PSC without cholangiocarcinoma showed aneuploidy of nuclear DNA. The high prevalence of DNA aneuploidy indicates that DNA cytometry may be helpful in separating malignant strictures from benign. The role of DNA aneuploidy for the detection of premalignancy remains to be defined. Aneuploidy in the epithelium of large bile ducts was found in two patients (12%). These two patients were both men, 51 and 52 years old at the time of liver transplantation, and there were no signs of malignancy in the explanted livers. Both patients suffered from UC and one was operated on with colectomy at 14 years of age and the other underwent colectomy at 48 years of age due to presence of colorectal dysplasia. This is interesting since PSC patients with concomitant UC and colorectal cancer/dysplasia have been suggested to run an increased risk of developing cholangiocarcinoma (19). Whether the aforementioned two patients would have developed cholangiocarcinoma later or not could only be speculated on, and prospective studies are needed. If aneuploidy of DNA precedes malignant transformation, regular brushings at ERCP with assessment of DNA ploidy could be a complementary approach to identify PSC patients at increased risk of developing cholangiocarcinoma (61, 62). We are currently evaluating this approach.

The increased risk of hepatobiliary carcinoma in PSC is demonstrated in the study of the largest cohort in the world including 604 PSC patients. This represents approximately 2/3 of all PSC patients in Sweden (Paper V). The frequency of hepatobiliary carcinoma in this study was 13%. One third of the PSC patients with hepatobiliary malignancy already had the cancer at time of PSC diagnosis, which is in line with findings in an earlier study by Ahrendt et al

(34). However, some of these cancer patients may not have an underlying PSC. Therefore, we made a careful evaluation to avoid including patients without PSC or secondary sclerosing cholangitis. We looked for signs of PSC, including presence of IBD, previous history of raised serum alkaline phosphatase levels, and portal fibrosis or cirrhosis in the liver biopsy. In all patients, an underlying PSC was likely, and the frequency of IBD in this group was not different from that of the cohort as a whole. Despite the large number of patients included in Paper V, we were unable to confirm earlier data of an increased risk for cholangiocarcinoma in PSC patients with previous colorectal cancer/dysplasia (19). The reason for this can be an underestimation of cases with colorectal dysplasia, since no re-evaluation of biopsies from the colon was made in the present study, and only patients having undergone colectomy due to dysplasia were considered as having dysplasia.

For the first time, it is shown that patients with PSC seem to have an increased risk not only for cholangiocarcinoma and colorectal cancer but also for pancreatic carcinoma, the risk being increased 14 fold (Paper V). Patients with PSC have an increased risk of developing cancer in tissues exposed to chronic inflammation – in the biliary tract, and in the colorectal mucosa if the patient also suffers from ulcerative colitis (10, 19, 20, 97, 98). Changes at cholangiography consistent with chronic pancreatitis are seen in 15 to 46% of all patients with PSC (23, 24) (25) and it was recently shown that patients with PSC frequently show hyperamylasemia (99). In addition, chronic pancreatitis without association to PSC is a known risk factor for the development of pancreatic carcinoma (100, 101). Chronic inflammation therefore seems to play an important role in the development of malignancy in patients with PSC. The overlap between distal hepatobiliary carcinoma and pancreatic carcinoma is however a diagnostic dilemma. It is sometimes difficult to differentiate primary pancreatic carcinoma from cholangiocarcinoma, especially at an advanced tumour stage and spread. Diagnostic mistakes

and detection bias can therefore not be ruled out (histologically, pancreatic carcinomas are usually of ductal type and from a purely microscopic point of view impossible to differentiate from cholangiocarcinomas).

Conclusions

In clinical settings, it is difficult to distinguish PSC patients with end stage disease from those with liver malignancy. Only careful sectioning of the liver either at transplantation or at autopsy discloses the tumour, in most cases. PSC patients being current or former smokers are at an increased risk of developing bile duct carcinoma.

Criteria for biliary duct dysplasia can be agreed on and the entity recognised in liver biopsies. The strong association of biliary duct dysplasia with cholangiocarcinoma in PSC suggests that such dysplasia can be used as a marker for current or developing malignancy. Increased bile duct proliferation may be used as a surrogate marker for premalignancy in PSC. In addition, PSC patients with cholangiocarcinoma more often display biliary epithelial dysplasia than those with end-stage PSC, indicating that the dysplasia may represent a pre-cancerous stage in PSC. p53 mutation seems to be a late event in the cancerous change, since no p53 expression was found in the pre-malignant areas of non-tumorous bile ducts.

The major cause of death in PSC is cancer. PSC patients also run an increased risk of developing pancreatic carcinoma. The risk of hepatobiliary malignancy is increased 161 by (95% CI, 120–210) times. One third of the PSC patients already have hepatobiliary malignancy at the time of PSC diagnosis. The incidence rate of hepatobiliary carcinoma is constant after the first year following the PSC diagnosis being 1.5% per year.

There is thus a need for spotting PSC patients with incipient neoplasia before manifest cancer occurs and identifying patients at increased risk of developing cholangiocarcinoma. False positive diagnosis of malignancy must always be avoided since cholangiocarcinoma may disqualify a patient from a life saving liver transplantation. The challenge for the future is to understand the factors involved in the increased neoplastic potential in PSC and to try to interfere in the carcinogenic process.

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