K-ras mutations in the bile of patients with primary sclerosing cholangitis

S Kubicka, F Kühnel, P Flemming, B Hain, N Kezmic, K L Rudolph, M Manns, P N Meier

Abstract

***Background and aims*—The development of cholangiocarcinoma (CCC) is a complication of primary sclerosing cholangitis (PSC). To date, no reliable factors have been described which can define those PSC patients at high risk for the development of CCC and the clinical diagnosis of CCC in PSC patients is difficult. Therefore, molecular markers of cholangiocarcinogenesis, such as K-ras mutations, may improve the early diagnosis of CCC or the timing of liver transplantation.**

***Methods*—K-ras mutations were analysed by enriched polymerase chain reaction/restriction fragment length polymorphism in the bile fluid of 56 PSC patients and 20 patients with other cholestatic diseases. To assess the value of K-ras mutations as a risk factor for cholangiocarcinogenesis, patients were prospectively investigated over a mean period of 31.5 months.**

***Results*—In contrast with the control group, 17 (30%) patients with PSC revealed K-ras mutations in bile fluid. The mean Mayo score was not significantly different between PSC patients with (mean score 0.70) and without (mean score 0.13; p=0.2) K-ras mutations. In contrast with the group of PSC patients without K-ras mutations, four CCCs and two dysplasia were diagnosed in the group of patients with K-ras mutations during the follow up investigation (p<0.001).**

***Conclusions*—Our results indicate that K-ras mutations in bile fluid of PSC patients represent frequent early events during cholangiocarcinogenesis. However, most of the PSC patients with K-ras mutations remained tumour free after a long follow up investigation which is in agreement with the fact that these mutations are not specific for malignancy but may also occur in normal bile duct mucosa or in dysplasias. Therefore, analysis of K-ras mutations in bile should not be used for diagnosis of CCC in PSC patients. However, the results of our prospective follow up investigation indicate that K-ras mutations in bile fluid of PSC patients have to be considered as risk factors for the development of CCC which may have implications for the timing of liver transplantation.**

(*Gut* 2001;**48**:403–408)

Keywords: K-ras mutations; cholangiocarcinogenesis; molecular diagnosis

Department of Gastroenterology and Hepatology, Medizinische Hochschule Hannover, Germany
S Kubicka
F Kühnel
B Hain
N Kezmic
K L Rudolph
M Manns
P N Meier

Department of Pathology, Medizinische Hochschule Hannover, Germany
P Flemming

Correspondence to: Professor MP Manns, Department of Gastroenterology and Hepatology, Medizinische Hochschule Hannover, Carl Neubergstraβe 1, 30625 Hannover, Germany. manns.michael@mh-hannover.de

Accepted for publication 25 September 2000

Primary sclerosing cholangitis (PSC) is a chronic cholestatic disease of unknown aetiology, characterised by inflammation and progressive obliterative fibrosis of the intra- and extrahepatic bile ducts.[1–3] The clinical course of PSC is variable and unpredictable. The disease is often progressive and the majority of patients develop end stage liver disease. Large studies have shown an estimated median survival of approximately 12 years in symptomatic patients.[4–6] One commonly used treatment option with acceptable results for patients with end stage PSC is orthotopic liver transplantation (OLT). The one year survival rates of PSC patients after OLT have been reported to range from 71% to 88%.[7–10] Patients with PSC should be considered for liver transplantation if the estimated survival rate of the natural course of the disease is lower than after OLT. On the other hand, there is evidence that the most advanced stages of PSC appear to have an increased mortality and morbidity associated with OLT. Thus optimal timing of OLT, based on disease specific scores[4 6 11 12] or on the Child-Pugh classification,[13] is important to improve outcome and decrease morbidity.

One complication of PSC is the development of biliary malignancies. The time from the initial diagnosis of PSC until recognition of cholangiocarcinoma ranges from 1 to 25 years.[14–17] The prognosis of PSC patients with cholangiocarcinoma is poor: the estimated median survival after diagnosis of the tumour is 5–7 months.[12 16] In particular, in patients who are considered for liver transplantation, the incidence of cholangiocarcinomas is high. Several studies revealed cholangiocarcinomas in 10–36% of PSC patients at the time of liver transplantation.[6 7 18 19] In a retrospective study from our centre, cholangiocarcinomas were diagnosed in 10 of 48 (21%) explanted livers from PSC patients.[20] In most of these studies the majority of tumours were clinically silent and were only detected by the pathologist in the explanted livers, thus highlighting the difficulties in the clinical diagnosis of cholangiocarcinomas in PSC patients.

However, the diagnosis of cholangiocarcinoma dramatically decreases the prognosis of PSC patients after OLT. In particular, tumour recurrence after liver transplantation is common.[1 6 20] As liver function remains constant in

Abbreviations used in this paper: CCC, cholangiocarcinoma; OLT, orthotopic liver transplantation; PSC, primary sclerosing cholangitis; ERC, endoscopic retrograde cholangiography; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

many patients with PSC, the development of cholangiocarcinomas should also be considered as an indication for OLT. But to date no reliable factors have been described which can define those PSC patients at high risk for the development of cholangiocellular carcinomas and the established prognostic disease scores are of limited use in predicting the risk of cholangiocarcinogenesis.

Some studies revealed a high incidence of bile duct dysplasia in patients with PSC and cholangiocarcinomas, consistent with the concept of multistage carcinogenesis.[21 22] In colorectal cancer one frequent molecular event before the morphological occurrence of dysplasia is mutation of the K-ras oncogene.[23] However, there is evidence that the incidence of K-ras mutations is high not only in biliary tract cancer but also in biliary tract dysplasia.[24]

As K-ras codon 12 mutations represent excellent candidates for the molecular diagnosis of gene alterations in several body fluids,[25–30] we hypothesised that similar mutations may also be detected in bile fluid of PSC patients and may be used for early tumour diagnosis or for establishing a risk factor for cholangiocarcinogenesis.

Experimental procedures

PATIENTS AND BILE FLUID SAMPLES

Fifty six patients with PSC and 20 patients with other benign cholestatic liver diseases (benign bile duct stenosis after OLT (n=11), choledocholithiasis (n=5), liver cirrhosis (n=3), Budd-Chiari syndrome (n=1)) were included in the study. The diagnosis of PSC was based on accepted criteria, including histological, biochemical, and radiological findings.[1 2] Malignant tumour at the time of presentation of patients was excluded by ultrasound, tumour marker CA19-9, endoscopic retrograde cholangiography (ERC), and bile duct cytology.

Clinical variables were obtained by review of the patients' charts at the Medizinische Hochschule Hannover. The onset of PSC was defined as the time of first presentation of abnormal liver function test consistent with a diagnosis of PSC.

Bile juice was obtained during ERC. Bile fluid (2 ml) was centrifuged at 12 000 rpm for 15 minutes. The pellet was washed twice with phosphate buffered saline and subsequently stored at −80°C.

The explanted livers of PSC patients undergoing OLT were intensively investigated by a pathologist. Tissue from the bile tract system with macroscopic evidence of hyperplasia or cholangiocarcinoma was fixed in formalin and embedded in paraffin wax.

DNA EXTRACTION AND ENRICHED PCR-RFLP

Tissue sections (5 μm) were cut from each of the tissue blocks. Adjacent sections were stained with haematoxylin-eosin to confirm the histological diagnosis. Genomic DNA from the tissue specimen or from the bile pellets was extracted using the QIAamp tissue kit (Quiagen Inc. Valencia, USA) according to the manufacturer's instruction. To avoid DNA contamination, each of the samples was processed independently.

Enriched polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed as described previously[31 32] with minor modifications. Amplification with Taq polymerase was performed in 100 μl reaction mixtures containing 2 units of polymerase, 100 pmol of each primer, 2mM Mg^{2+}, 60 mM KCl, 10 mM Tris-HCl (pH 8.8), and dNTP (dATP, dCTP, dGTP, dTTP) at 200 μmol. The reaction mixture was overlaid with 75 μl of mineral oil and subjected to amplification (Landgraf). Each cycle consisted of 94°C for one minute, 55°C for 90 seconds, and 72°C for 90 seconds. The first PCR comprised 20 cycles; 5 μl of the PCR product were digested with 2 units of BstNI (New England Biolabs Inc., Beverly, USA) in a volume of 20 μl for 12 hours. Wild type fragments cleave to yield 114 base pairs whereas mutant fragments yield 143 base pairs; 1/1000 of the first digest was used as a template for the second PCR of 30 cycles. During this step the 143 base pair fragments with the ras codon 12 mutations were selectively amplified. The sequences of the primers in the first PCR were: (A) 5' - ACTGAATATAAACTTGTGGTAGTTG GACCT-3'; and (B) 5'-TCAAAGAATGGT CCTGGACC-3'. In the second PCR, instead of primer (B), primer (C) was used: (C) 5'-GCATATTAAAACAAGATTTAC-3'.

A second BstNI digest with 20 μl of the second PCR product was performed. Subsequently, the digest was separated by electrophoresis on a 2% agarose gel.

DNA SEQUENCING

If a ras codon 12 mutation specific band was noted in a sample by agarose gel electrophoresis, 1 μl of the second PCR product was used for subcloning the DNA fragment in a plasmid using the TA cloning kit (Invitrogene, Carlsbad, USA) according to the manufacturer's instructions. DNA sequencing of the K-ras genes in plasmids was performed with primer C using the sequenase 2.0 kit (Amersham Life Science, Cleveland, Ohio, USA). To rule out PCR generated artefacts, all K-ras mutations were confirmed by repeating PCR-RFLP and DNA sequencing.

MAYO NATURAL HISTORY MODEL FOR PRIMARY SCLEROSING CHOLANGITIS

The clinical data for calculation of the Mayo risk scores were obtained at the time of ERC where bile fluid was collected for molecular analysis. The risk score was calculated as 0.003×age (years), +0.54×$\log_e$ (total bilirubin (mg/dl)), −0.84×albumin (g/dl), +0.54×$\log_e$ (aspartate transaminase (IU/l)), +1.24×variceal bleeding (yes=1; no=0), as described previously.[33]

STATISTICAL ANALYSIS

The χ^2 test and Fisher's exact test were used to compare differences between proportions. A difference with a p value equal to or less than 0.05 was considered significant. Fisher's exact

Table 1 Incidences of K-ras mutations in patients with primary sclerosing cholangitis (PSC) with or without ulcerative colitis (UC)

PSC	PSC and UC	PSC without UC	Control group
17/56	7/20	10/36	0/20
30%	35%	27%	0%
		p=0.39	

UC, ulcerative colitis diagnosed by clinical and endoscopic evaluation. Control group comprised patients with benign bile duct strictures after liver transplantation (n=11), choledocholithiasis (n=5), liver cirrhosis (n=3), and Budd-Chiari syndrome (n=1).

Table 2 Scores for the Mayo natural history model in patients with primary sclerosing cholangitis (PSC) with regard to K-ras mutations

	Mean value		Range
PSC with K-ras mutations (n=15)	0.70	p=0.2	−1.2–3.92
PSC without K-ras mutations (n=29)	0.13		−1.6–4.03

test was used to compare the occurrence of K-ras gene mutations and various factors. p values <0.05 were considered statistically significant

Results

INCIDENCE OF K-ras CODON 12 MUTATIONS IN BILE JUICE OF PATIENTS WITH PRIMARY SCLEROSING CHOLANGITIS

As K-ras codon 12 mutations can be detected in several body fluids by a sensitive PCR-RFLP, we hypothesised that molecular analysis of K-ras mutations in the bile of PSC patients may be used for the early diagnosis of cholangiocarcinomas in these patients. Thus we prospectively investigated the bile fluid of PSC patients, obtained during retrograde cholangiography, for K-ras mutations at codon 12.

Fifty six patients with PSC without clinical evidence of biliary tract malignancies were included in the study. The incidence of ulcerative colitis and K-ras mutations in the bile of patients is shown in table 1.

Mutation patterns included GGT to GTT transversion (n=7), GGT to GAT transition (n=6), GGT to AGT transition (n=3), and GGT to GCT (n=1) transversion. The mutation spectrum resembled the base substitutions of the K-ras gene described in cholangiocarcinomas by other investigations.[24 31 32] There was no significant difference in the incidence of K-ras mutations between PSC patients with or without ulcerative colitis (table 1). As a control, the bile juice of 20 patients with cholestatic diseases without evidence of PSC or biliary tract malignancy were analysed for K-ras mutations. In contrast with the bile juice from PSC patients, no K-ras codon 12 mutations were observed (table 1). As 30% of PSC patients without clinical evidence of cholangiocarcinomas showed K-ras mutations, we hypothesised that these gene alterations do not reflect undetectable cholangiocarcinomas but are frequent early molecular events in bile duct mucosa of PSC patients which may promote the development of cholangiocarcinomas.

Table 3 Prognostic significance of K-ras mutations for the development of bile duct dysplasias and cholangiocarcinomas (CCC)

	Mean follow up period (months)	CCC	Dysplasia	Dysplasia + CCC
PSC with K-ras mutations (n=17)	34.6	4	2	6
		p=0.006	p=0.082	p<0.001
PSC without K-ras mutations (n=39)	30.2	0	0	0

Several natural history models have been described to asses the prognosis of PSC patients to improve the timing of liver transplantation[4 6 11–13 33] but none of these models contained factors to assess the risk of carcinogenesis. Thus we investigated the correlation between the established Mayo natural history model score of PSC[33] and the occurrence of K-ras mutations. Reliable clinical data for this risk score, obtained at the time of ERC, were available for 44 of 56 PSC patients. Although there was no significant difference (p=0.2) in the disease specific score between patients with or without K-ras mutations in bile, the incidence of K-ras gene alterations tended to be higher in patients with advanced PSC (table 2).

FOLLOW UP STUDY OF PATIENTS WITH PSC AND K-ras GENE MUTATIONS IN BILE JUICE

K-ras mutations in the bile of PSC patients may reflect either molecular events in normal mucosa, premalignant bile duct lesions, or early stages of cholangiocarcinomas. The high incidence of these gene alterations in PSC patients without clinical evidence of cholangiocarcinomas indicates that K-ras mutations occur often in normal mucosa or in premalignant bile duct lesions and have to be considered as a risk factor for tumour development. To asses the role of K-ras mutations in bile as a risk factor for PSC patients, we performed a follow up investigation. During the follow up periods all patients were systematically investigated with ultrasound and assessment of the tumour marker CA19-9 every three months. In cases with evidence of tumour growth at ultrasound or CA19-9, a CT scan and ERC with bile duct cytology were also performed.

In the group of patients without K-ras mutations no cholangiocarcinoma was clinically diagnosed during the follow up period (mean follow up duration 30.2 months; range 6–54). During follow up, 15 PSC patients without K-ras mutations underwent liver transplantation. The explanted livers were investigated by a pathologist. The histology of the explanted livers revealed only the typical inflammatory bile ducts of PSC. There was no evidence of bile duct dysplasia or malignancy.

Also, PSC patients with K-ras mutations in bile fluid were prospectively investigated. The mean follow up period was 34.6 months (range 1–44). In this group of patients, two cholangiocarcinomas were clinically diagnosed by imaging techniques on ultrasound and CT scan during the follow up period. The malignancies were confirmed by cytology. The intervals between detection of K-ras mutations in bile and clinical diagnosis of cholangiocarcinomas were 14 months and 36 months, respectively (tables 3, 4).

During follow up, four patients with K-ras mutations in bile fluid underwent OLT. In two cases the explanted livers revealed dysplasias of large bile ducts and in two other cases inciden-

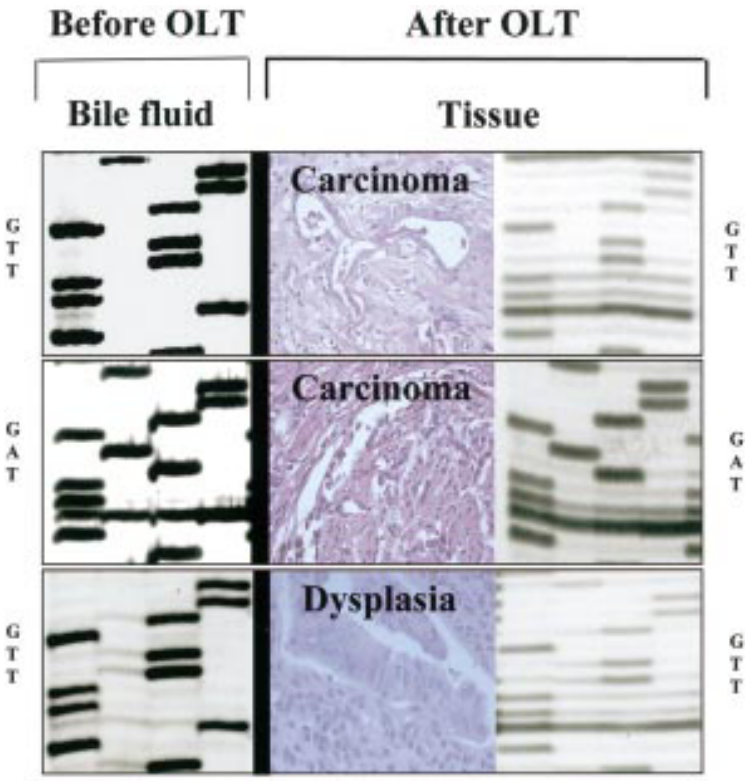

Figure 1 Tissues of two cholangiocarcinomas and two dysplasias of large bile ducts were available for analysis of K-ras mutations at codon 12 by PCR-RFLP and DNA sequencing after orthotopic liver transplantation (OLT). Three of the K-ras mutations initially found in bile fluid of patients with primary sclerosing cholangitis at the time of inclusion in the study (left) were confirmed in the corresponding tissues of two cholangiocarcinomas and one dysplasia after OLT (right).

tal cholangiocarcinomas were detected by the pathologist (fig 1). In one of these patients the interval between molecular analysis of a K-ras mutation in bile and the diagnosis of cholangiocarcinoma was only one month (table 4). Therefore, it is likely that the malignant tumour was already present at the time of ERC where bile fluid was collected. Although this carcinoma was only incidentally detected during liver transplantation, the tumour was at an advanced stage with local peritoneal metastases.

Tissues of two cholangiocarcinomas and two dysplasias were available for molecular analysis of K-ras mutations after OLT. Three of the K-ras mutations initially found in bile fluid of patients were confirmed in the corresponding tissues, as shown in fig 1.

Discussion

One known risk factor for the development of biliary tract cancer is PSC. Although many biliary tract carcinomas complicating PSC can be detected by imaging methods such as computed tomography, cholangiography, ultrasonography, and magnetic resonance,[34] there is an urgent need for further tools to improve the early diagnosis of cholangiocarcinomas in patients with PSC. The serum tumour markers CA19-9 and CEA may be helpful for detection of a cholangiocarcinoma in a patient with PSC.[22 35] In addition, FDG-PET may have potential in the diagnosis of biliary carcinomas in these patients and may facilitate the decision of whether or not to accept a PSC patient on a waiting list for liver transplantation.[36] However, the early diagnosis of biliary cancer in PSC patients remains difficult. Hence one aim of our study was to evaluate molecular analysis of K-ras mutations as a new method for the early diagnosis of cholangiocarcinomas in PSC patients. In our study, K-ras mutations were frequently observed in bile fluid of PSC patients. However, most patients with K-ras mutations in bile remained cancer free for more than 12 months after analysis of the K-ras gene mutation. As cholangiocarcinomas are very fast growing tumours with a poor prognosis, it is obvious that the sources of most of the K-ras mutations were not malignant tumours. Moreover, our study confirmed earlier observations that K-ras mutations can also be found in bile duct dysplasias, which are considered as premalignant lesions in PSC patients. These results indicate that K-ras mutations in PSC patients often represent early molecular events in normal mucosa or in dysplasias and are not specific signs of malignancy. Therefore, molecular analysis of K-ras mutations in the bile of PSC patients would seem to be of limited use for early diagnosis of cholangiocarcinomas.

As a consequence of these results we considered that the molecular diagnosis of K-ras mutations may be useful for establishing a molecular risk factor for carcinogenesis in PSC patients. In fact, our prospective investigation demonstrated that significantly more cholangiocarcinomas or dysplasias were diagnosed in the group of PSC patients with K-ras mutations in bile fluid during the mean follow up period of 31.5 months (p=0.002). Recently, a prospective study in patients with chronic pancreatitis investigated whether K-ras mutations

Table 4 Characteristics of patients with primary sclerosing cholangitis (PSC) who developed cholangiocarcinoma or bile duct dysplasia during the follow up investigation

Age	*First PSC diagnosis*	*K-ras mutation*	*Follow up investigation*	*Remarks*
45 y	1974	7/97	9/98 Carcinoma	Diagnosis of cholangiocarcinoma by CT scan and cytology. Normal CA19-9; T4N1M0.
36 y	1996	1/96	1/99 Carcinoma	Diagnosis of cholangiocarcinoma by CT scan and cytology. Normal CA19-9; T4N1M0.
31 y	1996	5/96	6/96 Carcinoma	Incidental diagnosis of cholangiocarcinoma during OLT. CA19-9: 762 kU/l; T1N0M1.
34 y	1993	7/96	8/97 Carcinoma	Incidental diagnosis of cholangiocarcinoma during OLT. Normal CA19-9; T1N0M1.
34 y	1989	4/96	7/97 Dysplasia	Diagnosis of bile duct dysplasia in explanted liver (OLT 7/97).
39 y	1983	4/97	5/97 Dysplasia	Diagnosis of bile duct dysplasia in explanted liver (OLT 5/97).

CT, computed tomography; OLT, orthotopic liver transplantation.

in duodenal fluid could identify high risk patients for the development of pancreatic carcinomas.[37] Contrary to our results, K-ras gene mutations in this study were not directly relevant to the development of pancreatic neoplasm. However, the risk of pancreatic cancer in patients with chronic pancreatitis is much lower than the risk of biliary cancer in PSC patients,[38] which may explain the different results obtained in our study regarding the prognostic value of K-ras mutations. To demonstrate the prognostic relevance of K-ras mutations in the bile of patients with chronic pancreatitis, a longer follow up study may be necessary.

In three cases K-ras mutations of bile fluid were confirmed in tissues with cholangiocarcinomas and dysplasia after liver transplantation. However, we failed to confirm all mutations found in bile in the corresponding tissues. This may be explained by methodical limitations, or may reflect the mulicentric development of cholangiocarcinomas in PSC patients.

In three of the four cholangiocarcinomas, the interval between molecular diagnosis of a K-ras mutation in bile and diagnosis of cholangiocarcinoma was longer than 11 months. Considering the poor prognosis of cholangiocarcinomas, detection of K-ras gene alterations in bile fluid in these patients either reflected a precancerous lesion (dysplasia) or a very early tumour stage. Although several studies demonstrated that incidental cholangiocarcinomas do not affect patient survival after liver transplantation,[39–41] the prognosis of these patients is difficult to estimate and depends in particular on tumour stage. However, it seems reasonable to assume that liver transplantation performed at the time of diagnosis of the K-ras mutations in bile fluid would have improved the prognosis of these three patients. But it remains speculative whether the prognosis of all PSC patients with K-ras mutations in bile fluid could be improved if they underwent transplantation earlier. Although our study indicates that K-ras mutations in bile fluid of PSC patients are poor prognostic factors for the development of dysplasia and/or cholangiocarcinomas, the optimal timing of liver transplantation of these patients should be investigated by further prospective studies.

Our study demonstrated that mutation of codon 12 of K-ras is a frequent early event in the carcinogenesis of PSC associated cholangiocarcinomas. K-ras mutations at codon 12 are not specific for malignant bile duct tumours in PSC patients and may often appear before the occurrence of morphological signs of dysplasia or malignancy. As a consequence, molecular analysis of K-ras mutations in bile fluid, obtained by ERC, seems of little use for early diagnosis of cholangiocarcinomas in PSC patients. However, a significant number of PSC patients with K-ras mutations developed cholangiocarcinomas or dysplasias during a follow up investigation in contrast with a group of patients without K-ras mutations. Thus K-ras codon 12 mutation in bile fluid appears to be a prognostic factor for PSC patients which may be important for the timing of liver transplantation.

We thank Monique Hörning for secretarial assistance. The research was supported by a grant from the Deutsche Forschungsgemeinschaft Sonderforschungsbereich 265, project C4A.

1 Chapman RWG, Arborgh BAM, Rhodes JM, *et al.* Primary sclerosing cholangitis: review of its clinical features, cholangiography and hepatic histology. *Gut* 1980;**21**:870–7.
2 Wiesner RH, LaRusso NF. Clinicopathologic features of the syndrome of primary sclerosing cholangitis. *Gastroenterology* 1980;**79**:200–6.
3 Lee YM, Kaplan MM. Primary sclerosing cholangitis. *N Engl J Med* 1995;**332**:924–33.
4 Wiesner TH, Grambsch PB, Dickson ER, *et al.* Primary sclerosing cholangitis: natural history and prognostic factors, and survival analysis. *Hepatology* 1989;**10**:430–6.
5 Broome U, Olsson R, Loof L, *et al.* Natural history and prognostic factors in 305 Swedish patients with PSC. *Gut* 1996;**38**:610–15.
6 Farrant JM, Hayllar KM, Wilkinson ML, *et al.* Natural history and prognostic variables in primary sclerosing cholangitis. *Gastroenterology* 1991;**100**:1710–17.
7 Marsh JW Jr, Iwatsuki S, Makowka L, *et al.* Orthotopic liver transplantation for primary sclerosing cholangitis. *Ann Surg* 1988;**207**:21–5.
8 Krom RAF, Wiesner RH, Rettke SR, *et al.* The first 100 liver transplantations at the Mayo Clinic. *Mayo Clin Proc* 1989; **64**:84–94.
9 Markus BH, Dickson ER, Grambsch PM, *et al.* Efficacy of liver transplantation in patients with primary biliary cirrhosis. *N Engl J Med* 1989;**320**:1709–13.
10 Langnas AN, Grazi GL, Stratta RJ, *et al.* Primary sclerosing cholangitis: the emerging role for liver transplantation. *Am J Gastroenterol* 1990;**85**:1136–41.
11 Dickson ER, Murtaugh PA, Wiesner RH, *et al.* Primary sclerosing cholangitis: refinement and validation of survival models. *Gastroenterology* 1992;**103**:1893–901.
12 Wiesner RH, Porayko MK, Dickson R, *et al.* Selection and timing of liver transplantation in primary biliary cirrhosis and primary sclerosing cholangitis. *Hepatology* 1992;**16**: 1290–9.
13 Shetty K, Rybicki L, Carey WD. The Child-Pugh classification as a prognostic indicator for survival in primary sclerosing cholangitis. *Hepatology* 1997;**25**:1049–53.
14 Aadland E, Schrumpf E, Fausa O, *et al.* Primary sclerosing cholangitis: a long-term follow up study. *Scand J Gastroenterol* 1987;**22**:655–64.
15 Rosen CB, Nagomey DM. Cholangiocarcinoma complicating primary sclerosing cholangitis. *Semin Liver Dis* 1991;**11**:26–30.
16 Rosen CB, Nagorney DM, Wiesner RH, *et al.* Cholangiocarcinoma complicating primary sclerosing cholangitis. *Ann Surg* 1991;**213**:21–5.
17 Broome U, Löfberg R, Veress B, *et al.* Primary sclerosing cholangitis and ulcerative colitis: evidence for increased neoplastic potential. *Hepatology* 1995;**22**:1404–8.
18 Stieber AC, Marino IR, Iwatsuki S, *et al.* Cholangiocarcinoma in sclerosing cholangitis. The role of liver transplantation. *Int Surg* 1989;**74**:1–3.
19 Miros M, Kerlin P, Walker N, *et al.* Predicting cholangiocarcinoma in patients with primary sclerosing cholangitis before liver transplantation. *Gut* 1991;**32**:1369–73.
20 Nashan B, Schlitt HJ, Tusch G, *et al.* Biliary malignancies in primary sclerosing cholangitis: timing for liver transplantation. *Hepatology* 1996;**23**:1105–11.
21 Martins E, Fleming KA, Garrido MC, *et al.* Superficial thrombophlebitis, dysplasia, and cholangiocarcinoma in primary sclerosing cholangitis. *Gastroenerology* 1994;**107**: 537–42.
22 Bergquist A, Glaumann H, Persson B, *et al.* Risk factors and clinical presentation of hepatobiliary carcinoma in patients with primary sclerosing cholangitis: a case control study. *Hepatology* 1998;**27**:311–16.
23 Fearon ER, Vogelstein B. A genetic model for colorectal tumourigenesis. *Cell* 1990;**61**:759–67.
24 Ajiki T, Fujimori T, Onoyama H, *et al.* K-ras gene mutation in gall bladder carcinomas and dysplasia. *Gut* 1996;**38**: 426–9.
25 Tada M, Omata M, Kawai S, *et al.* Detection of ras gene mutations in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma. *Cancer Res* 1993; **53**:2472–4.
26 Caldas C, Hahn SA, Hruban RH, *et al.* Detection of K-ras mutations in the stool of patients with pancreatic adenocarcinoma and pancreatic ductal hyperplasia. *Cancer Res* 1994;**54**:3568–73.
27 Iguchi H, Sugano K, Fukayama N, *et al.* Analysis of ki-ras codon 12 mutations in the duodenal juice of patients with pancreatic cancer. *Gastroenterology* 1996;**110**:221–6.
28 Villanueva A, Reyes G, Cuatrecasas M, *et al.* Diagnostic utility of K-ras mutations in fine-needle aspirates of pancreatic masses. *Gastroenterology* 1996;**110**:1587–94.
29 Furuya N, Kawa S, Akamatsu T, *et al.* Long-term follow-up of patients with chronic pancreatitis and K-ras gene mutation detected in pancreatic juice. *Gastroenterology* 1997;**113**: 593–8.
30 Castells A, Puig P, Mora J, *et al.* K-ras mutations in DNA extracted from the plasma of patients with pancreatic

carcinoma: diagnostic utility and prognostic significance. *J Clin Oncol* 1999;**17**:578–84.
31 Levi S, Urbano-Ispizua A, Gill R, *et al.* Multiple K-ras codon 12 mutations in cholangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res* 1991;**51**:3497–502.
32 Watanabe M, Asaka M, Tanaka J, *et al.* Point mutation of K-ras gene codon 12 in biliary tract tumours. *Gastroenterology* 1994;**107**:1147–53.
33 Kim WR, Poterucha JJ, Wiesner RH, *et al.* The relative role of the Child-Pugh classification and the Mayo natural history model in the assessment of survival in patients with primary sclerosing cholangitis. *Hepatology* 1999;**29**:1643–8.
34 Campbell WL, Ferris JV, Holbert BL, *et al.* Biliary tract carcinoma complicating primary sclerosing cholangitis: evaluation with CT, cholangiography, US, and MR imaging. *Radiology* 1998;**207**:41–50.
35 Ramage JK, Donaghy A, Farrant JM, *et al.* Serum tumour markers for the diagnosis of cholangiocarcinoma in primary sclerosing cholangitis. *Gastroenterology* 1995;**108**:865–9.
36 Keiding S, Hansen SB, Rasmussen HH, *et al.* Detection of cholangiocarcinoma in primary sclerosing cholangitis by positron emission tomography. *Hepatology* 1998;**28**:700–6.
37 Furuya N, Kawa S, Akamatsu T, *et al.* Long-term follow-up of patients with chronic pancreatitis and K-ras gene mutation detected in pancreatic juice. *Gastroenterology* 1997;**113**:593–89.
38 Karlson BM, Ekbom A, Josefsson S, *et al.* The risk of pancreatic cancer following pancreatitis: an association due to confounding? *Gastroenterology* 1997;**113**:587–92.
39 Goss JA, Shackleton CR, Farmer DG, *et al.* Orthotopic liver transplantation for primary sclerosing cholangitis. A 12-year single center experience. *Ann Surg* 1997;**225**:472–81.
40 Jeyarajah DR, Klintmalm GB. Is liver transplantation indicated for cholangiocarcinoma? *J Hepatobiliary Pancreat Surg* 1998;**5**:48–51.
41 von Schonfeld J, Lange R, Bug R, *et al.* Liver transplantation in a 29-year-old patient with gallbladder carcinoma complicating primary sclerosing cholangitis. *Z Gastroenterol* 1998;**36**:977–81.