Detection of Ki-*ras* gene point mutations in bile specimens for the differential diagnosis of malignant and benign biliary strictures

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Abstract

Background and aim—The present study was undertaken to determine if detection of Ki-*ras* gene point mutations in bile specimens could differentiate between benign and malignant biliary strictures.

Patients—Bile specimens were obtained from 117 patients exhibiting a stricture of the main bile duct, the nature of which was assessed by cholangiography, histology, and follow up.

Methods—DNA from frozen bile specimens was extracted, amplified, and tested for codon 12 point mutations of Ki-*ras* gene using sequence specific oligonucleotide hybridisation and mutant allele specific amplification.

Results—DNA amplification was successful in 110/117 bile specimens (94%). Detection of Ki-*ras* gene mutations in bile specimens was positive in 24.4% (22/90) of patients with malignant strictures, in 31.4% (22/70) when only primary malignant tumours were considered, and in 4% (1/25) of patients with benign strictures. Of the 49 patients with histological specimens obtained before surgery, the sensitivity of histology, Ki-*ras* mutation analysis, and combined methods was 59.2%, 28.6%, and 73.5% respectively.

Conclusions—Our study showed that Ki-*ras* mutations may be detected in about one third of bile specimens from patients with primary tumours invading the main bile duct. Detection of such mutations appears to be specific and may help to differentiate between benign and malignant biliary strictures.

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Determining the cause of a stricture of the main bile duct, either benign or malignant in nature, is a prerequisite for treatment. The diagnosis of malignant biliary strictures rests on the identification of tumour cells obtained using various methods. Percutaneous fine needle aspiration requires the presence of a tumour mass: however, this can be visualised by ultrasonography or computer assisted tomography (CT) in only 40% of hilar cholangiocarcinomas.[1] Cytology performed directly on bile allows detection of malignant cells in only 6–26% of cancer cases.[2 3] Cytology after endobiliary brushing, and histology on biopsies, give somewhat better results with a higher sensitivity in cases of pancreatic tumours compared with cholangiocarcinomas.[4] However, although endobiliary brushing can be performed in up to 90% of biliary strictures, biopsies can be obtained in only 61% of cases.[5–8] These two methods allowed us to detect malignant cells in 35% and 43% of malignant biliary strictures, respectively, in a large prospective study.[6] The use of serum markers of malignancy is of little help. Ca 19-9 is not specific for malignancy in associated cholangitis,[9 10] and serum anti-p53 antibodies are detectable in only 5% of cholangiocarcinomas.[11] Therefore, new methods, such as molecular biology and image cytometry,[12] are required to improve the differential diagnosis of benign and malignant biliary strictures before surgery.

Mutations of the Ki-*ras* oncogene have been described in several human carcinomas, including pancreas and bile duct cancers.[13 14] These point mutations appear to be of biological significance in the complex process of cell transformation.[15] Therefore, when detected, they provide additional information on malignancy. Such point mutations mainly reside in the first two nucleotides of codon 12, making their detection by polymerase chain reaction (PCR) feasible. In carcinomas of the pancreas visualised at ultrasonography, Ki-*ras* gene mutations were detected in 64% of the specimens obtained by fine needle aspiration, with no false positive results.[16] Amplification of DNA from bile specimens is also possible, revealing point mutations associated with malignant strictures of the main bile duct.[17 18] However, the usefulness of detecting Ki-*ras* mutations has not yet been evaluated in a large prospective series of patients with benign and malignant biliary strictures.

We have assessed the value of detecting codon 12 point mutations of the Ki-*ras* gene in DNA extracted from bile specimens for the differential diagnosis of malignant and benign biliary strictures.

Materials and methods

PATIENTS
Patients were referred to Edouard-Herriot Hospital, a tertiary care unit for endoscopy and

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Abbreviations used in this paper: MASA, mutant allele specific amplification; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; SSOH, sequence specific oligonucleotide hybridisation; SSC, standard saline citrate; UTP, uridine 5'-triphosphate; TTP, thymidine 5'-triphosphate; CT, computed tomography.

radiology, for treatment of malignant or benign extrahepatic biliary strictures. Prospectively, 117 unselected patients were included in the study, between March 1994 and January 1997.

FINAL DIAGNOSIS
Diagnostic methods
The nature of the biliary strictures was assessed by a combination of the following methods: (i) radiological imaging including ultrasonography, CT scan, and cholangiography; (ii) cytology or histology on specimens obtained by endoscopic brushing or biopsies and, when possible, by percutaneous fine needle aspiration, depending on the morphology of the strictures and the presence and localisation of a tumour mass; (iii) surgery; and (iv) medical history and follow up.

Malignant strictures
Patients with suspected malignant strictures were divided into three groups. Group 1 included patients in whom the diagnosis of malignancy was considered certain. Carcinoma was proved at histology on specimens obtained at surgery or by non-surgical techniques. Group 2 included patients with a suspected diagnosis of malignancy; histological diagnosis was not available but other data obtained by cholangiography or other imaging systems were in keeping with a diagnosis of malignancy, which was confirmed by medical follow up showing progressive deterioration of the patient's general status leading to death. Group 3 consisted of patients who were excluded from the final analysis for different reasons. For them, cholangiography and other imaging methods indicated malignant strictures but histological samples were not available. Moreover, their general status was either unchanged or altered but they were still alive or lost at follow up by the end of the study.

Benign strictures
The stricture was considered benign when data obtained by cholangiography and other imaging methods suggested a diagnosis of benign stricture, and when the patient's general status was preserved.

BILE SAMPLES AND DNA EXTRACTION
Fresh bile (1 ml) was collected in tubes at the time of drainage and immediately frozen in liquid nitrogen. For DNA extraction, 0.5 ml of lysis buffer (0.5 M Tris HCl, pH 9.0, 16 mM EDTA, 10 mM NaCl containing 0.5% sodium dodecyl sulphate (SDS), and 0.1 mg/ml of proteinase K) was added to the bile sample and incubated overnight at 50°C. DNA was purified by two successive extractions, first by addition of phenol-chloroform-isoamyl alcohol (25:24:1) and second by addition of chloroform-isoamyl alcohol (24:1). DNA was then precipitated by adding three volumes of ethanol in the presence of 0.1 M CH_3COONa.

DNA AMPLIFICATION
PCR followed by PCR-mutant allele specific amplification (PCR-MASA) were performed with primers identical to those described previously.[19] The first PCR allows amplification of the sequence of Ki-*ras* gene coding for the first exon. To avoid false positive reactions by amplification of previous PCR products, deoxyuridine 5'-triphosphate (dUTP) was used instead of deoxythymidine 5'-triphosphate (dTTP), leading to new PCR products containing dU. The first PCR was performed in 1× PCR buffer (Eurobio, Les Ulis, France) with 10–100 ng of target DNA, 2 mM $MgCl_2$, 180 µM of each dNTP, 0.8 µM of each primer, 1 U of Taq DNA polymerase (Eurobio), and 0.1 U of uracyl-N-glycosylase (HK-UNG, Epicentre Technologie, USA) in a final volume of 50 µl. Each sample was first incubated at 37°C for 20 minutes and then denatured at 95°C for five minutes. DNA was amplified by 40 successive cycles (20 seconds at 94°C, 40 seconds at 55°C, and one minute at 72°C). PCR products were analysed by electrophoresis through a 2% agarose gel in Tris acetate-EDTA buffer (Sigma, St Quentin Fallavier, France) containing 0.5 µg/ml of ethidium bromide.

PCR-MASA was performed on previously amplified DNA by 40 successive cycles of 30 seconds at 95°C, 50 seconds at 62°C, and 50 seconds at 72°C. The reaction mixture contained 1× PCR buffer (Eurobio), 2 mM $MgCl_2$, 100 µM of each deoxyribonucleotide triphosphate (dNTP), 1 U of polymerase enhancer (Perfect Match, Stratagene, Montigny-le-Bretonneux, France), and 1 U of Taq DNA polymerase (Eurobio) in a final volume of 25 µl. Codon 12 point mutations of the first or second nucleotide were screened with two different mixtures of primers. Each set contains one common antisense primer and three discriminating sense primers at 0.35 µM except for primers exploring the mutations G12C (GGT→TGT) and G12D (GGT→GAT) at 0.45 and 0.50 µM, respectively. PCR-MASA products were subjected to electrophoresis through a 2% agarose gel containing 0.5 µg/ml of ethidium bromide. When DNA was amplified, giving a band of the expected size, three additional PCR-MASA were performed using the first PCR product as the starting material. Each was carried out with a single set of primers for discriminating each possible point mutation. Final analysis of the PCR-MASA products was carried out by agarose gel electrophoresis, as above.

SEQUENCE SPECIFIC OLIGONUCLEOTIDE HYBRIDISATION (SSOH)
The first PCR product of each sample was dot blotted onto positive nylon membranes (Appligene, Illkirch, France) and hybridised with radiolabelled oligomer probes. The membranes were prehybridised in 0.05 M phosphate buffer, pH 7.0, 0.1 M NaCl, 0.1% SDS, and 300 µg/ml of denatured herring sperm DNA for one hour at 54°C, and then hybridised overnight with ^{32}P labelled oligonucleotide probes recognising either wild-type Ki-*ras* gene or different Ki-*ras* mutants. Each oligonucleotide was labelled with $3.7×10^6$ Bq of [^{32}P] labelled γ ATP ($16.6×10^{13}$ Bq/mmol; ICN pharmaceuticals, Orsay, France) and T4

polynucleotide kinase. Membranes were rinsed at room temperature in 3× standard saline citrate (SSC) buffer, 0.1% SDS, and then washed three more times in 3× SSC for 30 minutes, successively at 58°C, 60°C, and 62°C. Autoradiography was performed overnight at −80°C with an intensifying screen. In these conditions, no cross hybridisation was observed between mutated probes.

POSITIVE CONTROL
Six different synthetic DNAs, each with a single point mutation of either the first or second nucleotide of Ki-*ras* codon 12, were used as positive controls. They were prepared by PCR site directed mutagenesis. Each was included as a positive control in the PCR-MASA and SSOH analyses.

PRESENTATION OF RESULTS
The frequency of Ki-*ras* gene point mutations detected in bile specimens was evaluated in different subgroups: malignant strictures (patients from groups 1 and 2 combined) versus benign strictures; primary carcinomas (pancreas, gall bladder, bile duct, or ampulla) versus metastases of other tumours; and primary tumours that developed in the main bile duct (bile duct and ampulla carcinoma) versus tumours possibly invading the bile duct (gall bladder and pancreas carcinomas). Statistical analyses were performed using Fisher's exact test (Statview, Berkeley, California, USA). $p<0.05$ was considered significant.

Results
FINAL DIAGNOSIS
Malignant strictures
Imaging data suggested a malignant biliary stricture in 92 of 117 patients, 52 (56.5%) from group 1, 38 (41.3%) from group 2, and two (2.1%) from group 3. All patients in group 1 had definite cancer, as assessed by histology or cytology on specimens obtained before surgery (24 (46.2%)) and by laparotomy (28 (53.8%)). All patients in group 2 exhibited a progressive alteration of their general status until death, with a median follow up of 4 months (range 1–29 months). The two patients in group 3 (excluded from the analysis) exhibited imaging features in keeping with a malignant stricture. However, one was lost to follow up; the second suffered progressive deterioration of his general status with frequent attacks of cholangitis but was still alive after a follow up period of 18 months.

Benign strictures
The biliary stricture was considered benign in 25 of 117 patients. The final diagnosis was: chronic pancreatitis in six, bile duct lithiasis in eight, post-surgical stricture in four, sclerosing cholangitis in five, portal cavernoma in one, and papillary sclerosis in one. All patients in this group remained alive with a stable general status. Median follow up was 18 months (range 12–49 months).

AMPLIFICATION OF DNA FROM BILE SPECIMENS
Amplification of the 286 bp of Ki-*ras* DNA was successful in 110 of 117 bile samples (94%). The wild-type sequence was detected in all 110 cases by both SSOH and PCR-MASA. To determine the cause of the failure of DNA amplification in the remaining seven bile specimens (obtained from patients with one benign and six malignant strictures, respectively), control DNA extracted from HeLa cells was added to these samples before the first PCR. This control DNA could not be amplified, suggesting that inhibitors of PCR were present in these seven bile specimens, as already suggested for stools.[19]

POINT MUTATIONS OF Ki-*ras* IN DNA AMPLIFIED FROM BILE SPECIMENS
Table 1 shows the sensitivity, specificity, and positive and negative predictive values of Ki-*ras* point mutations detected in bile specimens for confirmation of malignant strictures. Figure 1 shows the 22 point mutations detected in bile specimens sampled from the 90 patients in groups 1 and 2 (24.4% sensitivity, 95% confidence interval 15.5–33.3%). Ki-*ras* point mutations were detected in 31.4% (22/70) of cases of primary tumours compared with 0/20 metastases of other tumours ($p<0.05$), and in 27.3% (9/33) of tumours developed in the main bile duct (bile duct and ampulla carcinomas) versus 35.1% (13/37) of cases of gall bladder or pancreas carcinomas (NS). One mutation was detected in the 25 bile specimens originating from patients with benign strictures (96.0% specificity, 95% confidence interval 88.3–100.0%). The patient had a jejuno-biliary anastomosis previously performed for complicated cholecystectomy. He was followed for 36 months after bile drainage and did not exhibit

Table 1 Sensitivity, specificity, and positive and negative predictive values of Ki-ras point mutation detected in bile specimens and non-surgical histological results (histology) in the diagnosis of malignant versus benign biliary strictures

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SSOH	17	96	94	15
PCR-MASA	24	96	95	23
SSOH and PCR-MASA	25	96	96	25
Histology	59	100	100	ND
Histology and Ki-*ras* mutations	73	96	97	ND

PPV, positive predictive value; NPV, negative predictive value; ND, not done.
SSOH, sequence specific oligonucleotide hybridisation; PCR-MASA, polymerase chain reaction-mutant allele specific amplification.
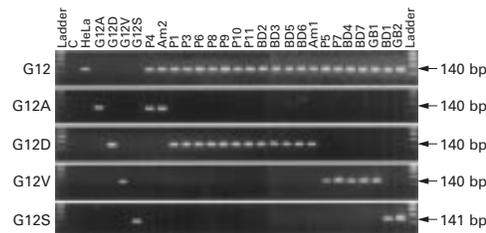

Figure 1 Gel electrophoresis analysis of the PCR-MASA products stained by ethidium bromide. Wild-type (G12) as well as mutated amplified DNA fragments are specified to the left. The origin of the amplified sample is indicated above (C, control without DNA; HeLa, HeLa cell DNA; G12A, G12D, G12V, and G12S, positive control DNA in which GGT codon 12 was changed for GCT, GAT, GTT, and AGT respectively; wild-type and mutated DNA originating from bile specimens of patients exhibiting pancreas (P), ampulla (Am), bile duct (BD), and gall bladder (GB) cancer.

any change in his general well being. He was therefore considered as having a benign biliary stricture. The predictive positive and negative values of Ki-*ras* mutation analysis for differentiating between benign and malignant biliary strictures were 96% and 25%, respectively.

PATIENTS WITH HISTOLOGICAL SPECIMENS OBTAINED BEFORE SURGERY

The diagnosis of malignant biliary strictures by non-surgical methods rests on examination of biopsies or cells obtained by endoscopy or percutaneous fine needle aspiration.[6 20] However, such analyses are not 100% reliable and give negative results even in cases of definite malignant strictures, as demonstrated later by surgical means.[6] The prevalence of Ki-*ras* point mutations was thus assessed in bile specimens from 49 patients with malignant strictures (groups 1 and 2) and with histological specimens obtained before surgery (endoscopic brushing in 16, endoscopic biopsies in 14, percutaneous cytology in 19). These histological specimens showed criteria for malignancy in 29 (59.2%) of the 49 cases. Ki-*ras* mutations were detected in bile specimens from 14 (28.6%) of these 49 cases, including seven of the 20 (35.0%) cases for whom the histological specimen was negative. In this group of 49 patients, the sensitivity of histology, Ki-ras mutation analysis, and combined methods were 59.2%, 28.7%, and 73.5%, respectively (table 1). The predictive negative value of histology and combined methods is not stated because histological specimens were available in only two patients with benign strictures.

COMPARISON BETWEEN SSOH AND PCR-MASA

Two G12A, 12 G12D, five G12V, and two G12S mutations were identified after PCR-MASA in bile samples from patients with malignant strictures. Concordance with the results given by the two methods was complete in all 15 cases that harboured a Ki-*ras* point mutation simultaneously detected by both methods. In seven cases, SSOH was not sensitive enough for detecting the point mutation found by PCR-MASA, irrespective of the nature of the mutation. One mutation identified by SSOH could not be screened by PCR-MASA (the sample was lost).

Discussion

The present study is the first large prospective series reporting the diagnostic power of Ki-*ras* mutation analysis in bile samples from patients with malignant and benign biliary strictures. DNA was extracted from bile specimens and amplified successfully by PCR in 94% of the 117 cases. Ki-*ras* gene point mutations of codon 12 were detected in one third of bile specimens from patients with primary tumours but in none of 20 bile specimens from patients with liver metastases. Moreover, in patients with histological specimens obtained before surgery, detection of Ki-*ras* mutations in bile specimens improved the diagnosis of malignancy over histology alone.

Detecting Ki-*ras* point mutations in bile samples relies primarily on the success of the first DNA amplification by PCR, which failed in up to 45% of cases in preliminary studies.[17 18] In our series, amplification was unsuccessful in only 6% of cases, probably not because of the absence of DNA in the sample but of the likely presence of DNA polymerase inhibitors that could not be eliminated from the bile specimens. When DNA amplification was successful, there was perfect concordance between SSOH and PCR-MASA regarding the type of Ki-*ras* mutation, but PCR-MASA was clearly more sensitive than SSOH. Interestingly, this method has no major technical difficulties and is fast enough to give results within 48 hours. Systematic use of dUTP instead of dTTP, together with treatment with uracyl N-glycosylase before the first PCR, eliminated false positives due to possible contamination by products of previous amplifications. Therefore, each point mutation depicted in our study was considered as existing in the original DNA sample.

Because of its high specificity, detection of tumour DNA in biological samples, in common with Ki-*ras* gene mutations in the present series, may represent an important tool for the diagnosis of malignancy in the future. The sensitivity of Ki-*ras* mutation analysis is however limited by the prevalence of these mutations in the tumour type under investigation, and is dependent on the presence of a sufficient amount of tumour cells in the biological sample used for the analysis. The prevalence of Ki-*ras* mutations, as assessed directly from tumour samples, was shown to be high in pancreatic and biliary tumours: 80–95% for pancreatic adenocarcinomas, 50–100% for cholangiocarcinomas, and 39–55% for gall bladder adenocarcinomas.[13 21–23] In cases of malignant biliary strictures, negative results obtained from Ki-*ras* mutation analysis of bile specimens may be explained in several ways. Firstly, as stated before, the tumour itself may present no Ki-*ras* mutation. Secondly, the tumour may reject a low amount of malignant cells in bile because of its scirrhous nature (for example, most cholangiocarcinomas) or because of extraluminal development (for example, some cancers of the pancreas). In particular, no mutations were detected in liver metastases from distant tumours, as previously reported,[18] which suggests that metastases compress rather than invade the main bile duct and do not reject a sufficient amount of malignant cells in bile. Several methods could be used to improve the detection of tumour DNA when drainage of potentially malignant bile duct strictures is scheduled. These are detection of different (codon 13 and codon 61) point mutations of the Ki-*ras* gene, search for DNA abnormalities different from Ki-*ras* mutations in bile specimens, and finally DNA amplification on samples obtained by brushing of the stricture itself instead of bile specimens.[13 22–24]

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