Long-term follow-up of patients with a clinically benign extrahepatic biliary stenosis and K-ras mutation in endobiliary brush cytology

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Background
K-ras mutations in endobiliary brush cytology are an early event in carcinogenesis and justify a suspicion of malignancy in patients with extrahepatic biliary stenosis. However, K-ras mutations have been detected in specimens obtained by brushing of clinically benign extrahepatic biliary stenosis. The aim of this study was to determine whether these findings represent an early or false-positive diagnosis of cancer.

Methods
Cytologic specimens were obtained by brushing in 312 consecutive patients with extra-hepatic biliary stenosis. Mutations in the K-ras oncogene were detected by an enriched polymerase chain reaction and allele-specific oligonucleotide hybridization assay. Eight patients with a K-ras mutation and a clinically benign extrahepatic biliary stenosis were followed.

Results
After a median follow-up of 65 months, 6 of the 8 patients were alive without evidence of malignancy. One patient died of congestive heart failure. The other patient died 60 months after the specimen was obtained, possibly because of chronic pancreatitis, although previously there had been suspicion of malignancy. Biopsy specimens from the papilla were negative for neoplasia and the K-ras analysis harbored the same mutation as previously found in the brush specimen.

Conclusions
Based on long-term follow-up, the K-ras mutations in all 8 patients with a clinically benign extrahepatic biliary stenosis must be considered as confirmed false-positives. Nevertheless, a false-positive result is infrequent. Therefore, patients with a positive K-ras mutation in biliary cytologic specimens obtained by brushing still require careful, continuing follow-up.
INTRODUCTION

Treatment strategy for stenosis of the extrahepatic bile duct is based on the determination of etiology, whether benign or malignant. A malignant stenosis is diagnosed by identification of tumor cells in tissue samples obtained by various means. One of the best methods for obtaining material from an undiagnosed bile duct stenosis and for differentiating malignant from benign causes is endoscopic retrograde cholangiopancreatography (ERCP).\(^1\) Cytologic confirmation is mandatory in making therapeutic decisions. Although the specificity of brush cytology for detecting malignant stenoses is high, reportedly up to 100%, its sensitivity remains low, in most studies ranging from 35% to 40%.\(^1,2\) Relatively new methods, such as molecular techniques, are required to improve the differential diagnosis of benign and malignant bile duct stenoses.

K-ras codon 12 mutations are an early and the most common event in carcinogenesis in the region of the head of the pancreas and can be detected in small samples.\(^3,4\) Several studies have found that K-ras codon 12 mutational analysis of cytologic specimens obtained by brushing at ERCP may help to differentiate between malignant and benign stenoses, and that this method is more accurate than conventional cytologic evaluation alone.\(^1,2,5,6\) However, the reported specificity of K-ras mutational analysis in cytologic brushings varies widely, which leaves its value open to question.\(^6,10\) In a large prospective study in our institution, K-ras mutations were detected in 11% of the patients with a clinically benign extrahepatic biliary stenosis (EBS).\(^2\) On one hand, this finding may indicate early detection of an incipient neoplastic lesion, as yet not otherwise evident; on the other, this result could be a true false-positive. Long-term follow-up of patients with clinically benign EBS and K-ras mutations in cytologic specimens from the bile duct is therefore needed to clarify this contradictory situation. This is a follow-up study of 8 patients with clinically benign EBS who harbored K-ras codon 12 mutations in their brush cytology specimens.

PATIENTS AND METHODS

Patients

From 312 previously described, consecutive patients with EBS, endobiliary brush cytology specimens were obtained prospectively at ERCP from January 1993 until February 1996.\(^2\) The results of conventional cytologic evaluation by light microscopy and K-ras codon 12 mutational analysis were compared and evaluated in relation to the final diagnosis based on histologic and/or clinical findings (table 1, figure 1). A final diagnosis could be made in 294 patients, of which 220 (75%) had a malignant and 74 (25%) a benign stenosis (figure 1). For this initial study all patients were followed for at least 12 months.

Eight of the 74 patients felt to have a clinically benign stricture had a K-ras codon 12 mutation identified in the cytologic specimen obtained by brushing. The current study concerns the follow-up of these 8 patients (table 1). Two patients had a diagnosis of chronic pancreatitis, 3 had a
<table>
<thead>
<tr>
<th>Procedure</th>
<th>Malignant Stenosis</th>
<th>Benign Stenosis</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Dx</td>
<td>n = 116</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Resection</td>
<td>n = 42</td>
<td></td>
<td></td>
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<tr>
<td>Autopsy</td>
<td>n = 7</td>
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<tr>
<td>Biopsy</td>
<td>n = 67</td>
<td></td>
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<tr>
<td>Clinical Dx</td>
<td>n = 104</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progressive disease</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Stable disease</td>
<td>n = 64</td>
<td></td>
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</tbody>
</table>

**Figure 1** Established final diagnosis, either clinical or tissue, in 294 of the 312 patients evaluated for extrahepatic biliary stenosis. 1: Gallstone in the gallbladder causing extrahepatic bile duct obstruction by external compression. 2: PSC, primary sclerosing cholangitis.

postsurgical stenosis, and 3 patients had primary sclerosing cholangitis. Six patients had a mutation for aspartic acid (nucleotide sequence GAT) at codon 12, the other 2 for alanine (GGT). Two of the 8 patients with a clinically benign stenosis harboring a K-ras mutation, both with a diagnosis of postsurgical stenosis, also had a positive cytology. At the time, these were felt to represent false-positive cytologic diagnoses because the two patients were not suspected clinically of harboring malignancy, and an explanation was sought in the repetitive stent insertion procedures in these patients. The cytologic specimens from all 8 patients were reevaluated by an outside independent expert cytopathologist who was unaware of the study results. For the current study, the observation period was from the point at which the initial brush specimen was obtained until December 31, 2000 (median 65 months, range 57-90 months). Patients were followed by contacting their primary care physicians for recent information on their health. When a patient was hospitalized during follow-up, all the data from the medical record were retrieved and scrutinized with emphasis on a putative diagnosis of malignancy. Both the former and the present study were approved by the Medical Ethical Review Committee of our institution. All participating patients provided informed consent.
Table 1 Results of cytology and K-ras mutational analysis with reference to final diagnosis

<table>
<thead>
<tr>
<th>Definitive diagnosis</th>
<th>Malignant stenosis</th>
<th>Benign stenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K-ras positive</td>
<td>K-ras negative</td>
</tr>
<tr>
<td>Positive</td>
<td>35</td>
<td>44</td>
</tr>
<tr>
<td>Negative</td>
<td>39</td>
<td>65</td>
</tr>
<tr>
<td>Suspect</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Insufficient</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>92</td>
<td>128</td>
</tr>
</tbody>
</table>

All patients were followed for 12 months. *Current studied group of patients

Materials

Cytologic specimens were obtained from the bile duct stenoses with a brush (GRBH-230-3-3.5, Wilson-Cook Medical Inc., Winston-Salem, N.C.). Four cytology smears from each patient were stained for routine cytologic evaluation. The remainder of the brush cytology specimen was suspended in 10 mL DNA buffer and fixed with 10 mL 100% ethanol. The suspensions were stored at 4°C for subsequent K-ras mutational analysis.

Methods

DNA isolation: One mL of each brush cytology suspension was used for DNA isolation. The protocol for extraction of DNA and the K-ras codon 12 mutational analysis has been described. K-ras mutational analysis: In this assay, DNA is subjected to polymerase chain reaction (PCR) amplification by using primers around codon 12. One of the primers generates a restriction enzyme recognition site with the wild type codon 12 sequence but not with the mutant codon 12 sequence. Digestion of the PCR products with the restriction enzyme is followed by a second round of amplification that then yields a PCR product enriched for K-ras codon 12 mutations. The resulting DNA fragments are denaturated and dot-blotted onto nylon membranes and subjected to allele-specific oligonucleotide (ASO) hybridization with radioactive labeled probes, specific for each possible K-ras codon 12 mutation, followed by autoradiography.

Cell suspensions with mutant to wild-type ratios of 1:100 and 1:1000 were used as positive controls in every PCR procedure. The suspensions were made of the human colon cancer cell line SW 480 with a homozygous GGT to GTT mutation at codon 12 of K-ras, and the human colon cancer cell line HT 29 with wild-type K-ras. Water was used as a control for contamination, placental DNA for nonspecific hybridization, and cloned DNA fragments with the 6 different K-ras codon 12 mutations and the wild-type codon 12 for specific hybridization. The above mutational analysis has been validated though comparison with sequence analysis.
The K-ras mutational analysis results were evaluated without reference to any information regarding the patient. All mutational analyses were performed in duplicate in separate experiments. In case of a discrepancy, a third analysis was performed. A result was called K-ras mutant positive if identical mutations were found in the duplicate analysis, and when the enrichment step for the mutation had been successful; that is, intensity of the "mutant" dot had increased after the digestion, whereas the wild-type dot had diminished or disappeared (figure 2). The sensitivity and specificity of this assay, assessed in a previous study, were, respectively, 42% (92/220) and 89%, and the positive and negative predictive values were, respectively, 92% and 34% (table 1).

On the available material from the biliary tract of the 8 study patients obtained during prolonged follow-up, K-ras mutational analysis was performed with the same assay.

Figure 2 Example of an autoradiogram of the K-ras mutational analysis of endobiliary brush cytology specimen obtained during ERCP: 7 nylon membranes, each hybridized with a different radioactive labeled oligonucleotide specific for the sequence of the wild-type codon 12 (left) and the 6 possible mutations. For each membrane, left lane is nonenriched PCR products and right lane mutant-enriched PCR products. A mutant specimen should create a weak signal in the nonenriched and a strong signal in the enriched columns because enrichment increases the proportion of mutant DNA. WT, Wild-type = glycine; Cys, cysteine; Ser, serine; Arg, arginine; Val, valine; Asp, aspartic acid; Ala, alanine. Row 1 contains the hybridization controls, DNA complementary to the labeled oligonucleotides. Rows 2 and 3 contain samples of, respectively, 1 and 5 μl of the microdissected material obtained by papillotomy of the patient who was suspected of having a pancreatic carcinoma. They harbor a gly (GGT) to asp (GAT) mutation at codon 12 of the K-ras gene. Row 4 is the control for contamination, water. Rows 5 and 6 are positive controls showing 1 cell with mutant codon 12, coding for the amino acid valine, mixed in 100, respectively, 1000 cells with wild-type codon 12 (1:100, respectively 1:1000). Row 7 is the control for amplification, placenta DNA.
RESULTS

Of the 8 patients with a K-ras mutation in the cytologic specimens obtained by brushing of a benign EBS, 6 were still alive without evidence of malignancy after a median follow-up of 68 months. These patients had been seen regularly by their primary care physicians who confirmed that none had any symptoms of obstructive biliary disease. The median overall survival of all 8 patients was 65 months.

One patient died in hospital of congestive heart failure without evidence of malignancy 56 months after the cytologic specimen had been taken and the final diagnosis of primary sclerosing cholangitis (PSC) was confirmed by obtaining biopsy specimens of the stenotic lesion. Autopsy was not performed.

The other patient died 60 months after the initial brush cytology was obtained. He had insulin dependent diabetes mellitus and intermittent episodes of jaundice with concomitant pain, presumably from chronic alcohol-induced pancreatitis. A lesion was found in the head of the pancreas on transabdominal US and EUS, but the results of fine needle aspiration (FNA) were repeatedly negative. A biliary stent was inserted endoscopically and exchanged repeatedly. Histopathologic evaluation of the most recent available material obtained at papillotomy 11 months before death revealed inflammation but not infiltrating carcinoma. The K-ras codon 12 mutational analysis performed on this tissue showed a mutation to aspartic acid (GAT), which was concordant with the mutation previously found in the brush cytology specimen (figure 2).

Six months before death this patient was hospitalized because of severe vomiting and weight loss. At endoscopy, it was impossible to pass the endoscope through the pylorus. Subsequent CT clearly demonstrated a stenosis in the duodenum with a small, deformed bulb, probably caused by recurrent duodenal ulcers in the past. Surgical exploration revealed portal hypertension with liver cirrhosis and chronic pancreatitis, but despite the suspicion of malignancy, there was no sign of a tumor. Subsequently, a gastroenterostomy and a choledochojunostomy with a Braun’s anastomosis were performed without resections. During the last months before his death, the patient again developed jaundice with concomitant weight loss, tiredness, and abdominal pain. Beside a cirrhotic liver and massive ascites, CT again demonstrated a mass in the pancreas. Because of the patient’s poor general condition, there were no remaining therapeutic options other than palliative measures. The official registered cause of death was “a benign disease, probably chronic pancreatitis.” Autopsy was not performed.

Re-evaluation of the cytology results for all 8 patients revealed the same findings: 2 of the 8 patients with a clinically benign stenosis harboring a K-ras mutation also had a positive cytology, whereas in the remaining 6 patients cytologic evaluation was negative.
DISCUSSION

This follow-up study demonstrated that K-ras codon 12 mutations found in cytologic specimens obtained by brushing at ERCP in 8 patients with clinically benign EBS should be considered confirmed false-positive results. After a median follow-up of more than 5 years, none of the 8 patients was proven to have malignancy. The overall 5-year survival in patients with pancreatic carcinoma is only 2%. Thus, any intervention based on the sole finding of a positive K-ras mutation in this group of patients with clinically benign disease would have been unjustified. Nevertheless, considering the complete series of 312 consecutive patients, although false-positive K-ras mutations may occur, they are infrequent; the positive predictive value of the test remains 92% and the specificity 89%. K-ras mutational codon 12 analysis can be considered supplementary to conventional light microscopy evaluation of brush cytology specimens obtained at ERCP for the diagnosis of malignant extrahepatic biliary stenoses, particularly those caused by pancreatic cancer. Hyperplastic duct lesions are frequently found together with cancer in the pancreas, and indeed there is evidence that ductal hyperplasia can progress to infiltrating carcinoma, that this lesion is a precursor to cancer, even though its natural history is unknown. This implies that K-ras mutational analysis can be used for the early detection of pancreaticobiliary carcinogenesis. Brat et al. reported 3 patients with precursor duct lesions who developed pancreatic cancer after intervals of 17 months to 10 years. In a study by Berthelemay et al. two patients developed pancreatic cancer, respectively, 18 and 40 months after the finding of a K-ras mutation in their pancreatic juice. In these patients the initial diagnosis was pancreatitis, a disease considered to commonly harbor precursor duct lesions and to be a condition that predisposes to pancreatic carcinoma.

The K-ras gene probably does not represent the unique link between chronic pancreatitis and pancreatic cancer. Although K-ras mutational analysis is a valuable adjunct to brush cytology, there is a wide variation in reported specificity. The specificity of 89% in our studies, based on data from as many as 312 consecutive patients with EBS, is one of the highest reported. The procedure that was used can therefore be considered as relatively reliable. Nevertheless, a diagnostic test with a specificity of 89% remains suboptimal, particularly in view of the major therapeutic consequences in case of a diagnosis of malignancy. Most false-positive results are described in studies of patients with chronic pancreatitis.

The clinical relevance of mutant K-ras in pancreatitis is presently still unknown. A longer follow-up period is needed to determine its significance because the cumulative risk of pancreatic cancer 20 years after the diagnosis of chronic pancreatitis is 4.0%. Although the follow-up period in the present study is 5 years, better test specificity could not be obtained by prolonging the follow-up for another 15 years because only 1 of the 2 patients with pancreatitis is still alive. In view of the cumulative risk of cancer after a diagnosis of chronic pancreatitis as mentioned above, it is debatable whether it was too early to conclude that the K-ras mutation found in the
patient with chronic pancreatitis who had died 60 months after the specimen was obtained is a confirmed false-positive result. The final clinical diagnosis of benign disease was based on the repeatedly negative fine needle aspirations, which revealed inflammation but not infiltrating carcinoma, and survival of more than 5 years after the K-ras positive brush specimen was taken. Although autopsy would have provided ultimate proof, in our opinion there is enough evidence to regard the K-ras mutation in this patient as a confirmed false-positive. The finding of the same K-ras mutation in material from the papillotomy site and the first brush cytology specimen does nevertheless underscore the reliability of the assay used (figure 2).

Two of the 3 patients with a clinically benign post-surgical stenosis and a K-ras mutation detected in the endobiliary brush specimen also had repeatedly positive cytologic findings for malignancy. As described in the initial study, despite a positive cytology these patients did not undergo resection. Although this is uncommon, the combination of a clinically benign course of the disease and the repeated exchange of stents in both patients, led to the decision against surgery. After 60 and 90 months both were still alive without evidence of malignancy. These were the only 2 patients with false-positive cytopathologic readings in the previous study of 312 consecutive patients with EBS, which made the specificity of this conventional test alone 98%. This is concordant with the reported specificity for definitive cytologic diagnosis. Although it may not be possible to differentiate high-grade dysplasia from malignancy by cytology, it becomes less likely that these positive cytologic specimens came from pancreatic duct lesions with high-grade dysplasia or in situ carcinoma, considering the length of follow-up. However, neither the length of time required for high-grade dysplasia to progress to cancer nor the proportion of lesions that undergo this transformation are known.

It is clear that duct hyperplasia does not always progress to invasive carcinoma during the life span of an average patient. For example, hyperplastic duct cells are found in the pancreas in the absence of cancer or chronic pancreatitis, that is, in the elderly as shown in autopsy studies. In the study of Tada et al., mutations found in these duct lesions differed from those found in pancreatic cancer. This does not correspond to our experience and it is our belief that the number of mutant alleles in the specimen may be more informative. A quantitative test for K-ras mutations has been developed and tested in lung tumor DNA samples. In this test ARMS allele specific amplification is coupled with real-time fluorescent detection of PCR products. The quantitative nature of the test makes it possible to set a threshold above which the specificity of the test is 100% for that particular patient cohort. This test may be more promising for clinical application in the future.

In conclusion, this follow-up study emphasizes that the results of K-ras analyses on endobiliary specimens obtained by brushing should be interpreted with caution. In all 8 patients with a clinically benign biliary stenosis followed long-term, the K-ras mutation in the endobiliary brush cytology specimen must be considered as confirmed false-positives. Nevertheless our reported
positive predictive value of 92%, and specificity of 89% in a consecutive series of 312 patients is relatively high. Therefore, patients with a positive K-ras codon 12 mutation require careful, continuing follow-up.

REFERENCES


