K-ras and p53 in cancer of the pancreas and extrahepatic biliary tract

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Introduction

Malignancies in the region of the head of the pancreas, including cancers of the pancreas, duodenum, ampulla of Vater, and distal common bile duct, are among the most deadly cancers. Surgical resection has emerged as an effective treatment for these malignancies, but surgery is effective only in patients with localized disease [1]. Palliative bypass without resection of the primary is generally the treatment of choice for patients with liver metastases at the time of laparotomy. Despite the clinical importance of identifying liver metastases, benign bile duct proliferations (BDPs) may mimic liver metastases, and clinical and histopathological judgement as to the nature of liver lesions encountered at the time of laparotomy is frequently in error [2,3].

The two most common benign BDPs in the liver are bile duct adenomas and bile duct hamartomas. Bile duct adenomas, also called benign cholangiomas, are well demarcated, subcapsular proliferations of bile ductules [4,5]. The ductules are typically lined by cuboidal to low-columnar epithelial cells. These cells lack significant nuclear atypia and have a low mitotic rate [4,5]. Vascular and lymphatic invasion are, by definition, not present. Bile duct hamartomas, also called von Meyenburg complexes, are frequently multiple and are characterized by a proliferation of ectatic bile ducts that often contain bile [6-8]. Unfortunately, metastases from adenocarcinomas of the region of the head of the pancreas can be very well differentiated, and it can be difficult to distinguish between a BDP and a metastatic adenocarcinoma [4,9]. For example, Allaire et al. reviewed 152 bile duct adenomas in the files of the Armed Forces Institute of Pathology and found that the contributing pathologist’s diagnosis for 35 of the 152 cases was metastatic adenocarcinoma and that adenocarcinoma was raised in the differential diagnosis in another 20 cases [4]. Thus, in over one-third of the cases reviewed, even with the benefit of histological examination, adenocarcinoma remained in the differential diagnosis. Clearly, new tests need to be developed to distinguish benign BDPs from metastatic adenocarcinomas in the liver [4,5,10,11].

Molecular biology techniques can be used to characterize a neoplasm, and the genetic alterations in a neoplasm can, in turn, be used to suggest that a population of cells originated from that neoplasm, even in cases in which the neoplastic cells are admixed with much larger numbers of non-neoplastic cells [12]. For example, mutations in K-ras have been used to identify cells shed from pancreatic cancers in pancreatic juice samples, in cytological preparations, and in stool and blood specimens [13-16]. K-ras is a particularly attractive target for determining whether a collection of cells arose from a peripancreatic primary primary for several reasons. First, most peripancreatic neoplasms harbor mutations in K-ras [17-20], suggesting that K-ras will be a sensitive genetic marker. Second, mutations in this oncogene are essentially limited to a single codon, and so a limited number of probes can be used to detect these mutations, greatly simplifying the analysis [17].

The purpose of this study was to determine whether molecular tests for activating point mutations in codon 12 of K-ras could be used to distinguish between benign BDPs and metastatic adenocarcinomas in patients with peripancreatic adenocarcinomas.

Materials and Methods

Patient Selection and Tumor Material

The files (from March 20, 1984, through October 7, 1995) of the Division of Surgical Pathology of The Johns Hopkins Hospital were searched for cases in which the terms pancreas, liver, and adenocarcinoma appeared. The pathological diagnoses from the cases identified in this search were then reviewed, and cases were selected in which the patient had 1) a liver biopsy that interpreted to show a BDP, a granuloma, or a metastatic adenocarcinoma and 2) an adenocarcinoma of the pancreas, duodenum, distal common bile duct, or ampulla of Vater. All available histopathological slides were re-
viewed, and the liver lesions were classified histologically into five groups as follows: 1) granulomas, 2) bile duct hamartomas, 3) bile duct adenomas, 4) metastases, and 5) scars with benign BDPs [4-8]. Clinical and follow-up information was obtained from the Pancreas Cancer Database in the Department of Surgery.

Detection of Mutations in K-ras
Formalin-fixed and paraffin-embedded tissue blocks of the primary carcinomas and of the liver lesions were evaluated for point mutations of codon 12 of the K-ras gene. All analyses were performed without the knowledge of histopathological diagnoses.

Areas containing the lesions to be evaluated were scraped off 5 μm microscopic slides and collected in DNA isolation buffers. DNA was isolated as described by Wright and Manos [21]. The protocol used to analyze the tissue for point mutations in codon 12 of K-ras has been described in detail elsewhere [22-24]. Briefly, DNA isolated from the clinical specimens was subjected to polymerase chain reaction (PCR) amplification using primers A (5'-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3') and D (5'-TCA TGA AAA TGG TCA GAG AAA CC-3'). A 1 μl aliquot of the first PCR was then subjected to a second round of amplification using the semi-nested primers A and B (5'-TCA AAG AAT GGT CCT GGA CC-3'). The resulting DNA fragments were then spot-blotted onto seven different nylon membranes, and each of these separate membranes was hybridized with an allele-specific oligonucleotide (ASO) probe for the wild-type K-ras sequence or for one of the six possible activating point mutations in codon 12 of K-ras [22-24]. Positive controls included cloned wild-type and mutant sequences, and no DNA was added in the negative controls [24]. All ASO analyses were performed in duplicate.

Sequencing K-ras
In selected cases the mutations identified by ASO hybridization were confirmed by direct sequencing. The sequence of codon 12 was determined by automated fluorescent DNA sequencing, using the dideoxy chain termination method [25]. Mutant-enriched PCR products were used for cycle sequencing. In the first round of amplification of K-ras codon 12, a mismatched primer (primer A) was used to generate a restriction site in PCR products with the wild-type sequence. After digestion of the PCR products with the restriction enzyme Mval, a second round of PCR amplification was performed using primers A and B, yielding a mutant-enriched PCR product [24]. The products were purified using the QIAEX gel extraction kit (QIAGEN, Chatsworth, CA) following the manufacturer's protocol and sequenced using primers A and B and the Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Foster City, CA) according to the manufacturer's manual. The sequencing reaction products were analyzed on 5% Long Ranger gel (FMC Bioproducts, Rockland, ME) on an ABI Prism 377 DNA sequencer (Perkin Elmer).

Selection of Additional Benign Bile Duct Proliferations
The results of our analysis of this initial series of patients prompted us to collect additional liver lesions so that the prevalence of K-ras mutations in benign BDPs could be estimated more accurately. Forty-five additional BDPs (bile duct adenomas and hamartomas) were collected from the files of the Department of Pathology of The University of Leiden, The Erasmus University in Rotterdam, The Academic Medical Center in Amsterdam, and The Johns Hopkins Hospital in Baltimore. These additional cases were selected solely on the basis that the patient had a histopathologically documented BDP that was not included in the first series. Most of these BDPs represent incidental findings not associated with a peripancreatic neoplasm. These 45 additional cases of BDPs were evaluated for mutations in codon 12 of the K-ras oncogene using the ASO hybridization technique.
Results

Forty-eight patients met the criteria for inclusion in the initial portion of this study. In 2 of these 48 patients, the only liver lesions available did not amplify in the K-ras analyses. These two cases were not included in further analyses. One of the forty-six remaining patients had two liver lesions, one of which did not amplify. This patient was included in the analyses because of the presence of a second liver lesion from the same patient that did amplify.

The forty-six patients ranged in age from 41 to 82 years (mean 64.9). Twenty-seven were male, and nineteen were female. Twenty-nine of the forty-six patients had an adenocarcinoma of the pancreas, ten an adenocarcinoma of the ampulla of Vater, three a duodenal adenocarcinoma, three a bile duct adenocarcinoma, and one a mucinous cystadenocarcinoma of the pancreas.

Fifty-four liver lesions were analyzed from the forty-six patients. Two patients had three separate liver lesions, and four patients had two separate liver lesions. The remaining forty patients had a single liver lesion. Thirteen of the fifty-four liver lesions were histologically classified as bile duct adenomas, fourteen as hamartomas (Figure 7), fourteen as granulomas, five as scars with benign BDPs, and eight as metastases [4,5].

During the period of time that these 46 patients were operated on at The Johns Hopkins, 911 patients underwent a pancreaticoduodenectomy for an epithelial malignancy at The Johns Hopkins Hospital. Thus approximately 5% of patients who underwent a pancreaticoduodenectomy at The Johns Hopkins Hospital for a peripancreatic carcinoma had an epithelial liver lesion identified at the time of surgery.

K-ras Mutational Status in Primary Neoplasms (Table 1)
The K-ras mutational status of the 46 primary carcinomas was determined by PCR and ASO hybridization. Activating point mutations in codon 12 of the K-ras oncogene were identified in 21 (72%) of the 29 pancreatic adenocarcinomas, in 4 (40%) of the 10 ampullary adenocarcinomas, in 1 (33%) of the 3 duodenal adenocarcinomas, and in 2 (67%) of the 3 bile duct adenocarcinomas. The mucinous cystadenocarcinoma of the pancreas was wild-type. In 15 of the cancers the normal GGT sequence (glycine) was mutated to GAT (aspartic acid), in 7 it was mutated to GTT (valine), in 4 it was mutated to CTT (arginine), and in 1 it was mutated to TGT (cysteine) and in 1 to GCT (alanine). A representative dot-blot is shown in Figure 8.

Mutations were not detected in the negative controls, and the appropriate mutations were detected in each of the positive controls (Figure 8).

K-ras Mutational Status of the Liver Lesions (Table 1)
The K-ras mutational status of 53 of the 54 liver lesions could be determined by ASO hybridization. Activating point mutations in codon 12 of K-ras were identified in all 8 of the liver metastases, in none of the 14 granulomas, and in 2 (6.5%) of the 31 benign BDPs with amplifiable DNA. The mutations in the eight liver metastases were identical to those in the patients' corresponding primary carcinomas.

Both histologically benign BDPs with mutations were bile duct adenomas. In one of these two bile duct adenomas, a GGT to GAT (aspartic acid) mutation was found, and the identical mutation was present in that patient's primary pancreas cancer. This patient's primary carcinoma and liver lesion were biopsied only, and the patient died 5 months later of disease. The liver lesion and the primary pancreatic carcinoma are illustrated in Figure 9, and histopathological review of the liver lesion by three independent pathologists confirmed that it met all histopathological criteria for a bile duct adenoma [4,5]. The second patient with a bile duct adenoma with a K-ras mutation had two separate BDPs. One was an adenoma, and it harbored a GGT to GTT (valine) mutation, whereas the second BDP was wild-type, as was this patient's primary pancreas cancer. This patient's primary carcinoma and liver lesions were biopsied only, and...
TABLE 1
K-ras mutations identified in the original series of 46 patients

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>Total</th>
<th>Wild-type (GGT)</th>
<th>Mutant (GAT)</th>
<th>Aspartic acid (GAT)</th>
<th>Valine (GTT)</th>
<th>Arginine (GTT)</th>
<th>Cysteine (TGT)</th>
<th>Alanine (GCT)</th>
<th>Not amplifiable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma of the pancreas</td>
<td>29</td>
<td>8</td>
<td>21</td>
<td>11</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma of the ampulla</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma of the duodenum</td>
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<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma of the distal common bile duct</td>
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<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Mucinous cystadenocarcinoma</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metastases</td>
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<td>0</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Granuloma</td>
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<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hamartoma</td>
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<td>13</td>
<td>0</td>
<td>1</td>
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<td>Scars with BDPs</td>
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</tr>
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</table>

the patient died 23 months later of disease. Again, histopathological review of the liver lesion with mutant K-ras by three independent pathologists confirmed that the liver lesion met all histopathological criteria for a bile duct adenoma [4,5].

Six patients had multiple liver lesions biopsied. As noted previously, one of the liver lesions from a patient with two lesions did not amplify, and one patient with two BDPs was found to have an adenoma with a GGT to GTT (valine) mutation, whereas the second BDP was wild-type. The multiple liver lesions examined from the remaining four patients were all wild-type.

All thirteen hamartomas with amplifiable DNA were wild-type, as were all five scars with BDPs.

K-ras Sequencing
In 11 cases the mutations identified by ASO hybridization were confirmed by direct sequencing. In all cases the direct sequencing confirmed the mutational status identified by ASO hybridization. A representative sequence analysis is shown in Figure 10.

Analyses of Additional Benign Bile Duct Proliferations
The results of this initial series of patients prompted us to examine additional histologically benign BDPs for mutations in codon 12 of the K-ras oncogene. Forty-five additional BDPs were therefore collected and analyzed by ASO hybridization. Three (6.7%) of these forty-five BDPs harbored activating point mutations in codon 12 of K-ras. Mutations were identified in 2 (6.1%) of the 33 bile duct hamartomas and in 1 (8.3%) of the 12 bile duct adenomas. In all three cases, the normal wild-type GGT was mutated to GAT. One of the two patients with a bile duct hamartoma with a K-ras mutation was surgically explored for a serous cystadenoma, and the other for a cholangiocarcinoma. The patient with a bile duct adenoma with mutant K-ras was explored because of a clinical suspicion of a Klatskin tumor. The primary neoplasms of these three patients were not analyzed.

Combined Series
A total of 101 liver lesions from 93 patients were therefore analyzed for mutations in codon 12 of the K-ras oncogene. In this combined series of patients, acti-
K-ras mutations in bile duct proliferations

Activating point mutations of K-ras were found in 3 (12%) of the 25 lesions classified as bile duct adenomas, in 2 (4.3%) of the 46 lesions classified as bile duct hamartomas, in none (0%) of the 14 lesions classified as granulomas, in none (0%) of the 5 lesions classified as scars with associated BDPs, and in 8 (100%) of the 8 lesions histologically classified as metastases of the liver. Three lesions classified as bile duct hamartomas did not amplify.

Discussion

Benign BDPs in the liver include bile duct adenomas and bile duct hamartomas. Well-differentiated metastatic adenocarcinomas can mimic both of these lesions, and conversely, benign BDPs can mimic metastatic adenocarcinoma [4,9]. It is therefore not surprising that benign BDPs have been misdiagnosed as metastases in patients with known malignant neoplasms [2,3,4,10,26]. The consequences of confusing these two processes in patients with a peripancreatic carcinoma can be great. Patients with a localized carcinoma could receive palliative instead of curative surgery, or conversely, patients with unresectable metastatic disease might undergo unnecessary radical surgery. Clearly, our ability to distinguish between these two processes needs to be improved.

K-ras oncogene mutations are an attractive target to distinguish benign BDPs from metastatic adenocarcinomas for a number of reasons. First, if both the primary and the liver lesions are available for analysis, one can directly compare the genetic alterations present in each, thereby reducing the need to rely on an observer's interpretation of phenotype [12]. Second, the majority of peripancreatic adenocarcinomas harbor K-ras mutations, suggesting that K-ras could be a sensitive marker for the presence of cells originating from a peripancreatic primary [17-20]. Third, K-ras mutations, in peripancreatic adenocarcinomas, are essentially restricted to a single codon, codon 12, and so a limited number of probes can be used to detect these mutations, greatly simplifying the analysis [17]. Finally, K-ras mutations have already been successfully used to detect cells shed from peripancreatic cancers. K-ras mutations have been used to detect pancreatic cancer cells in pancreatic juice samples, in cytological and histological preparations, and in stool and blood specimens [13-15,27-30]. Mutant K-ras, because of its prevalence and ease of detection, is an ideal genotypic marker of cells originating from a peripancreatic primary.

We examined the utility of molecular analyses for activating point mutations in codon 12 of K-ras in distinguishing benign BDPs from metastases. This study was conducted in two parts. In the first part we determined the K-ras mutational status of 48 primary carcinomas and 56 liver nodules from 48 patients who had a peripancreatic adenocarcinoma and who also underwent a liver biopsy. In two patients the only liver tissue available did not amplify, leaving 46 patients with 54 liver lesions for analysis. We found that all eight liver metastases harbored mutant K-ras, and in all eight of these cases the same mutation was present in the corresponding primary adenocarcinoma. This observation confirms that K-ras mutations can serve as a relatively sensitive genotypic marker for the presence of metastases from a peripancreatic primary.

Although K-ras mutations appear to be a sensitive marker for the presence of a metastasis, they may not be absolutely specific. For example, in the pancreas, noninvasive duct lesions have been shown to harbor K-ras mutations [27]. Indeed, in this initial series of patients we identified activating point mutations in codon 12 of K-ras in 2 (4.5%) of the 45 histologically benign liver lesions with amplifiable DNA. These two mutations do not appear to be the result of PCR errors. First, in both cases the PCR was repeated, and the same mutation was identified in the repeat PCR. Second, we did not encounter any PCR-induced codon 12 mutations in the wild-type controls or in the granulomas that were included in the analyses.

Both of the histologically benign BDPs with mutant K-ras were classified as bile duct adenomas. This finding suggests either that our histological classification of these two lesions was in error,
that the lesions were in fact very well differentiated metastases, or that bile duct adenomas may harbor activating point mutations in K-ras. It is unlikely that the two bile duct adenomas were simply misclassified for three reasons. First, three independent pathologists agreed on the histopathological diagnoses. Second, in both cases the primary carcinomas expressed carcinoembryonic antigen, whereas the lesions classified as bile duct adenomas did not. Third, in one of the two cases the primary peripancreatic carcinoma was wild-type, whereas the bile duct adenoma harbored a GGT to GTT (valine) mutation. Thus, it would appear that bile duct adenomas can, in fact, rarely harbor activating point mutations in K-ras.

To confirm this finding we determined the K-ras mutational status of an additional 45 benign BDPs. Three (6.7%) of these forty-five lesions harbored activating point mutations in K-ras. When one combines the two series of patients, K-ras mutations were found in all 8 metastases and in 5 (6.6%) of 76 benign BDPs (P < 0.001, Fisher's exact test). These 5 cases included 3 (12%) of 25 lesions classified as bile duct adenomas and 2 (4.3%) of 46 lesions classified as bile duct hamartomas. All 5 lesions classified as scars with benign BDPs and all 14 granulomas were wild-type.

These findings have several implications. First, the nature of bile duct adenomas and hamartomas has been debated with some authors suggesting that they are reactive processes [4], whereas others have suggested that they are neoplastic [5]. If one uses the definition of a neoplasm as a clone of cells with a mutation in a cancer-causing gene, then the finding of mutant K-ras in a minority of these lesions suggests that at least some are neoplastic and not reactive. This conclusion is supported by isolated case reports of malignant degeneration of bile duct adenomas [31-33]. Second, from a more practical point of view, although the finding of a K-ras mutation in a glandular lesion in the liver is suggestive of a metastasis, it is not diagnostic. Benign BDPs may rarely harbor activating point mutations in K-ras. Furthermore, because there are only a limited number of possible mutations at codon 12 of K-ras, benign BDPs in the liver and an unrelated peripancreatic cancer may rarely even harbor the same mutation.

In conclusion, the vast majority (>90%) of benign BDPs in the liver do not harbor activating point mutations in codon 12 of K-ras. In contrast, most metastases from peripancreatic primary adenocarcinomas will harbor these mutations. These results suggest that K-ras mutational analysis will be helpful in distinguishing a metastasis from a benign process. There is, however, some overlap in the mutational spectra of BDPs and pancreatic carcinomas. Additional, more specific, molecular markers will therefore be needed before molecular testing can be definitively used to distinguish benign BDPs from metastatic well-differentiated adenocarcinomas.

References

K-ras mutations in bile duct proliferations


To confirm this finding we drew on the Koga mutation strains of no additional A2 benign HCC's. Three of these Koga 2p-over cancers harbored a microsatellite gene mutation in K. S. When one examines the two series of patients, Korla's mutation were found in 73 cases, whereas only 5% (56/70) of the cases included 11.2% of the cases classified as the other genotype and 2.14% (4/18) of the cases managed to score for future carcinogenesis. All 27 were associated with benign HCC's and 90.14% associated with wild type.

These findings have several implications. First, the gene may act as an oncofactor in the development of HCC. If so, it may be a critical point in the process. Second, the finding of a microsatellite gene mutation in the DNA in the absence of a tumor would not necessarily mean that the disease is a gene-related one. However, the finding of a microsatellite gene mutation in the 2p-over cancers is more likely to mean that the gene is involved in the development of HCC. Therefore, the microsatellite gene mutation in the 2p-over cancers may be a critical point in the process of carcinogenesis.