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Prevalence of Activating K-ras Mutations in the Evolutionary Stages of Neoplasia in Intraductal Papillary Mucinous Tumors of the Pancreas

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Objective
The purpose of the study was to determine the prevalence of activating K-ras mutations in the pancreas of patients with intraductal papillary mucinous tumors (IPMT) and to analyze their relation to the degree of site-specific histopathologic abnormality.

Background
Intraductal papillary mucinous tumors of the pancreas have a biologic behavior that is significantly different from pancreatic ductal adenocarcinoma. Activating K-ras mutations, which may be important events in a multistage process of carcinogenesis, have been reported in IPMT.

Methods
Forty-six different histologic specimens (comprising normal pancreatic ducts, hyperplasia, low-grade dysplasia, high-grade dysplasia–carcinoma *in situ*, and carcinoma) from 16 patients with IPMT and 9 specimens from patients with pancreatic ductal adenocarcinomas were designated by a pathologist. Genomic DNA was extracted from paraffin-embedded tissue sections after microdissection. The K-ras gene was amplified by polymerase chain reaction and subjected to DNA sequencing.

Results
The K-ras mutations were detected in at least one specimen in 13 (81.2%) of 16 patients with IPMT. All mutations were found in codon 12. No codon 13 mutations were detected. The relative frequency of K-ras mutations in the different stages of IPMT was 16.7% in normal epithelium and papillary hyperplasia, 28.6% in low-grade dysplasia, and 57.1% in high-grade dysplasia–carcinoma *in situ* and invasive carcinoma. The K-ras mutations were detected in 6 (66%) of 9 pancreatic ductal adenocarcinomas.

Conclusions

The K-ras codon 12 point mutations are as frequent in IPMT as in ductal adenocarcinoma. A stepwise increase in the frequency of codon 12 mutations correlated with the stage of neoplastic evolution to cancer. This finding is consistent with an important role of K-ras gene mutations in the transformation from normal epithelium to invasive carcinoma in the majority of patients with IPMT.

Recent reports have concluded that mucinous ductal ectasia and intraductal papillary neoplasms, once considered different types of intraductal tumors, are clinically and pathologically identical. The term intraductal papillary-mucinous tumors (IPMT), encompassing both tumor types, replaces the separate identifications.[1-4] Intraductal papillary mucinous tumors are distinctly different from pancreatic mucinous cystic neoplasms (mucinous cystadenoma and mucinous cystadenocarcinoma) and from pancreatic ductal adenocarcinoma.[5,6] Mucinous ductal ectasia and intraductal papillary neoplasms differ histologically only in the degree of mucin production, as acknowledged in the most recent World Health Organization histologic classification of the tumors of the exocrine pancreas.[7] That classification distinguishes intraductal papillary mucinous carcinomas and IPMT with dysplasia but includes all intraductal tumors that formerly were described under different terms such as intraductal mucin-producing tumor,[8] intraductal mucin hypersecreting neoplasm,[9,10] pancreatic duct villous adenoma,[11] and mucin-producing tumor of the pancreas.[12,13] Although IPMT with a low degree of dysplasia are classified as borderline tumors, all IPMT appear to have malignant potential.[2,3] A recent study investigated criteria to predict malignant variants of mucin-producing IPMT and found that the presence of diabetes, size of the tumor, marked dilation of the main pancreatic duct, main pancreatic duct type tumors, and detectable large mural nodules are indicators of a malignant variant.[14] Compared to ductal adenocarcinomas of the pancreas, IPMT clearly have a more favorable natural history characterized by a much longer time line and by a much higher cure rate.[1-3] These differences are perhaps explained in part by the intraductal tumor location, which promotes early presentation with acute pancreatitis in 22% to 56% of cases.[1-3,12,15-17] Inasmuch as various oncogenes are now known to be involved in the pathogenesis of cancers[18] and of pancreatic carcinomas in particular,[19-21] the evolution of malignancy of IPMT and their decreased aggressiveness, compared to ductal adenocarcinomas of the pancreas, may be because of a different spectrum of genetic changes.

The K-ras gene is one of the three members of the ras gene family that codes for highly related 21-kd proteins with GTPase activity.[22,23] Point mutations in codons 12, 13, and 61 of K-ras result in expression of an altered protein capable of transforming cells into a malignant phenotype.[22] The prevalence of activating mutations in ductal adenocarcinomas of the pancreas has been found in different studies to be 56% to 95%.[19,21,24-34] However, the frequency of K-ras gene mutations is not well documented in IPMT, a much less common neoplasm. Although one study of five intraductal papillary neoplasms failed to detect activating K-ras mutations,[29] other studies have found these mutations in 31% to 86% at exon 1.[29,35-39] Because IPMT characteristically contain histologic lesions with different degrees of hyperplasia and neoplasia in different loci of the tumor, some of the variability in reported prevalence of K-ras mutations may stem from the method of tissue selection. The aims of this study are to determine the overall prevalence of activating K-ras exon 1 mutations in the pancreas of patients with IPMT and to correlate these changes with the histopathologic abnormality at the specific site at which the mutation is found. Such observations may illuminate the role of K-ras mutations in the evolution of carcinogenesis.

MATERIALS AND METHODS

Selection of Histologic Specimens and Histologic Criteria

Tissue sections from surgical specimens from 16 patients with IPMT who underwent pancreatic resections were reviewed by 2 pathologists (CCC, MP) in a blinded fashion to confirm the original histologic findings. There was no interobserver variability. The diagnosis of IPMT was based on previously published criteria.[7] Features assessed included determination of the proportions of cytologically benign, adenomatous, or malignant epithelium; the degree of ductal papillary hyperplasia; the relative degree of mucinous metaplasia and intraductal mucin of each specimen; the malignant invasion of the pancreatic

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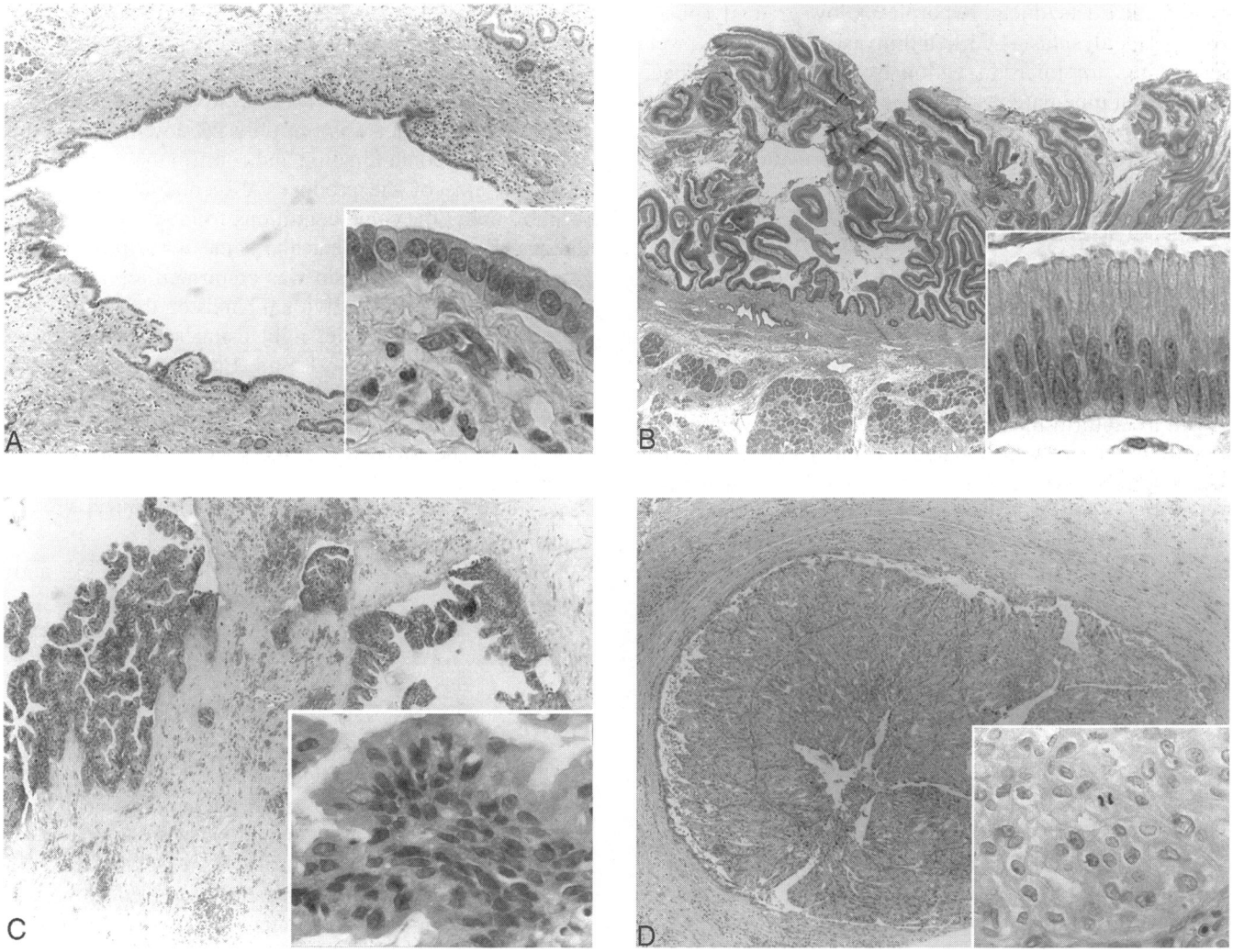

Figure 1. (A) Normal pancreatic duct (above left; magnification, ×80) lined by a single layer of cuboidal epithelium. The epithelium is serous in type and produces no mucin (inset; magnification, ×630). (B) Pancreatic duct showing papillary intraductal tumor with low-grade adenomatous dysplasia (above right; magnification, ×50) of the epithelium. Epithelial cells are tall columnar in shape, have increased nuclear size and chromaticity, and are pseudostratified but limited to the bottom halves of the cells (inset; magnification, ×630). (C) Intraductal papillary tumor with high-grade adenomatous dysplasia (below left; magnification, ×80) in which the nuclei are stratified into the luminal halves of the cells and mitotic activity is increased (inset; magnification, ×630). (D) Intraductal carcinoma with highly cellular papilla fitting the ductal lumen (below right; magnification, ×50). The cells show solid growth, pleomorphic cytology, and high mitotic activity (inset; magnification, ×400).

parenchyma; the pancreatic duct margin status; and the density of mitotic figures. Low-grade dysplasia was defined as adenoma, with nuclear crowding, hyperchromatism, and pseudostratification in the basal aspects of the cells (Fig. 1). High-grade dysplasia or carcinoma *in situ* (CIS) was diagnosed when cellular stratification to the apical aspects of the dysplastic cells occurred or when architectural changes such as cribriform epithelial growth was present (Fig. 1). Cytologic criteria for high-grade dysplasia–CIS included pleomorphism, increased nuclear to cytoplasmic ratios and prominent nucleoli. High-grade dysplasia lesions and CIS are grouped together in the current study. Invasive adenocarcinoma required the presence of infiltrating irregular neoplastic glands with a neoplastic stromal response. Specimens from nine patients with pancreatic ductal adenocarcinoma were reviewed in the same fashion to serve as a standard for cancer in the study.

After the slides from each individual patient were reviewed, different histologic lesions, comprising normal

pancreatic ducts, ductal hyperplasia, low-grade dysplasia, high-grade dysplasia–CIS, and invasive carcinoma, were designated for microdissection by a pathologist. Specimens from nine patients with pancreatic ductal adenocarcinoma were reviewed in the same fashion to serve as a control specimen for K-ras mutation detection.

Slide Preparation and Microdissection

New slides consisting of 10 to 20 serial sections (5 and 10 mm) from the above-mentioned paraffin-embedded tissue blocks were cut. The number of sections cut for microdissection depended on the size of the histologic lesion. The 5-mm sections were stained with hematoxylin–eosin to confirm that microdissected lesions were identical to the lesions designated by the pathologists. The unstained 10-mm sections were microdissected under a operating microscope using a ×30 to ×45 magnification. Disposable scalpels were used to prevent cross contamination. After microdissection, one of the slides of each lesion was stained with hematoxylin–eosin to confirm the correctness of the dissection. Fibrous septae and stromal reaction surrounding neoplastic components carefully were avoided to increase the cellularity of the selected area.

Genomic DNA Extraction

Microdissected specimens were deparaffinized in xylene. The tissue then was digested overnight with 200 mg/mL proteinase K at 50 C. Genomic DNA was extracted successfully with phenol, phenol–chloroform (1:1), and chloroform. DNA was precipitated with 2× volume of absolute ethanol followed by washing with 70% ethanol. Samples were centrifuged and DNA pellets resuspended in 25-mL sodium-free 1× Tris–ethylenediamine tetraacetic acid buffer. The DNA content was measured by optical density and purity verified by agarose electrophoresis.[40]

Polymerase Chain Reaction Amplification

A nested polymerase chain reaction (PCR) amplification of the K-ras gene was performed. The initial reaction mixture consisted of 200 ng DNA, 10 mmol/L Tris–hydrochloric acid, pH 8.3, 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride, 0.001% gelatin (wt/vol), 200 mmol/L of each deoxynucleotide triphosphate, and double-distilled water. The reaction mixture was heated to 95 C for 10 minutes to denature DNA. Primers (20 pmol final concentration) and 2.5 units Taq polymerase (Promega) then were added at 85 C. Thirty cycles of amplification with the first set of primers were carried out. One reaction cycle consisted of 94 C for 1 minute, 52 C for 1 minute, and 72 C for 1.5 minutes. The last cycle was followed by a 4-minute extension step at 72 C. The PCR product was run on a 2% low-melt agarose gel to exclude contamination and confirm the correct 157-base pair size of the product. A second 30-cycle PCR reaction under the same conditions followed, using a different antisense primer and the same sense primer. The second PCR product again was confirmed on a 2% low-melt agarose gel. The individual bands on the agarose gel were cut under ultraviolet light using sterile disposable scalpels and transferred to 1.5 mL Eppendorf tubes. DNA was extracted, purified, and quantitated as described above. The length of the second PCR product was 113 base pairs. The following primers were used in the PCR amplification:

K-ras: 5′ ACTGAATATAAACCTTGTGGTAG 3′ sense primer

K-ras: 5′ TCAAAGAATGGTCCTGGACC 3′ antisense primer I

K-ras: 5′ TTTACCTCTATTGTTGGATC 3′ antisense primer II

DNA Sequencing

The extracted PCR products were diluted to a concentration of 3 ng/mL and 10 mL per reaction used for sequencing. The dye terminator method with an ABI 373a sequencer was used. All samples were sequenced with the 5′- and the internal 3′-primers to confirm the K-ras sequence. Data were aligned and analyzed with Sequencher (gene codes).

Statistical Analysis

The difference in frequencies of K-ras oncogene mutations was analyzed by a Catanova test.[41] Differences of K-ras gene mutation frequencies between individual histologic groups in IPMT and between patients with IPMT and pancreatic ductal adenocarcinoma were calculated by Fisher's exact test.

RESULTS

Forty-six different histologic specimens from 16 patients with IPMT, comprising normal pancreatic ducts (12), hyperplasia (6), low-grade dysplasia (7), high-grade dysplasia–CIS (14), and carcinoma (7), were designated by the pathologist. One to six different lesions per patient were microdissected as indicated in Table 1. Examples of different microdissected lesions are shown in Figure 1. Nine different histologic specimens from nine patients

Table 1. DISTRIBUTION OF MICRODISSECTED LESIONS OF DIFFERENT HISTOLOGY IN 16 PATIENTS WITH INTRADUCTAL PAPILLARY MUCINOUS TUMORS OF THE PANCREAS*

Patient Number	Normal Duct	Hyperplasia	Low Grade Dysplasia	High Grade Dysplasia/CIS	Carcinoma	Codon 12 Sequences
1	wt	wt		wt/m	wt/m	GGT/CGT
2	wt			wt	m	GGT/CGT
3	wt		m		m/wt	GGT/TGT
4	wt/wt			m	wt	GGT/GAT
5					m	GGT/CGT
6	wt/wt	wt		m/wt		GGT/CGT
7	wt		wt/wt	m		GGT/GAT
8		wt	wt	m		GGT/CGT
9	wt/m	wt/m		wt		GGT/CGT
10	wt			m		GGT/CGT
11			wt	m		GGT/CGT
12				m		GGT/CGT
13				wt		GGT
14		wt		wt		GGT
15	m		m			GGT/CGT
16			wt			GGT

CIS = carcinoma *in situ;* PCR = polymerase chain reaction.
* Each distinct lesion is listed as either wild type (wt) or as heterozygose mutant (m) as determined by PCR and sequencing.

with ductal adenocarcinoma also were included in the study.

Activating K-ras mutations were detected in 13 (81.2%) of 16 patients with IPMT and in 17 (37%) of 46 different microdissected specimens, as listed in Table 1. In three patients, all analyzed lesions showed wild-type K-ras gene sequences in codons 12 and 13. All mutations were found in codon 12, and no codon 13 mutations were detected. Thirteen (76.5%) of 17 were GGT-to-CGT mutations, leading to a substitution of glycine by arginine; 11.8% were GGT-to-TGT mutations, replacing glycine by cysteine; and 11.8% were GGT-to-GAT mutations, replacing glycine by aspartate. All sequences with K-ras mutations demonstrated heterozygosity as shown in Figure 2. Activating mutations in pancreatic ductal adenocarcinomas were detected in 6 (66%) of 9. The activating codon 12 mutations detected in the ductal adenocarcinoma specimens were heterozygous; GGT/CGT was found in four specimens and GGT/GAT in two specimens. The difference in frequencies of K-ras mutations between patients with IPMT (81.2%) and ductal adenocarcinoma of the pancreas (66%) was not significant (Fisher's exact test). The relative frequency of K-ras mutations in the different stages of neoplasia, ranging from normal epithelium to invasive carcinoma, is shown in Figure 3. The stepwise increase in the frequency of activating K-ras mutations observed between the different groups of histologic lesions is statistically significant ($p = 0.0168$). The individual analysis of the subgroups did not show a significant difference between the groups of normal duct–hyperplasia specimens and low-grade dysplasia lesions, or between the low-grade dysplasia lesions and CIS–invasive carcinoma specimens with respect to K-ras gene mutation frequencies. There was no significant difference between the prevalence of K-ras mutations in IPMT carcinoma (57%) *versus* ductal adenocarcinoma (66%).

Twelve examples of histologically normal ducts were microdissected from 10 specimens of IPMT. Two of these were found to contain a K-ras gene mutation identical to that found either in an area of hyperplasia (CGT) or low-grade dysplasia (CGT) microdissected from a different locus on the same slide. Re-examination of the histologic appearance reconfirmed their cytologic normality (Fig. 1).

DISCUSSION

Intraductal papillary mucinous tumors of the pancreas are tumors with malignant potential but with a long natural history and a slow evolution to invasive carcinoma. Although locally advanced or metastatic cancers are not common, invasive carcinomas often are found in the resected specimens. In our own previously reported series, 11 (46%) of 23 patients with IPMT had invasive carcinoma, whereas an additional 42% of patients had CIS.[3] Despite this high prevalence of carcinoma, these patients have a favorable long-term survival rate after adequate

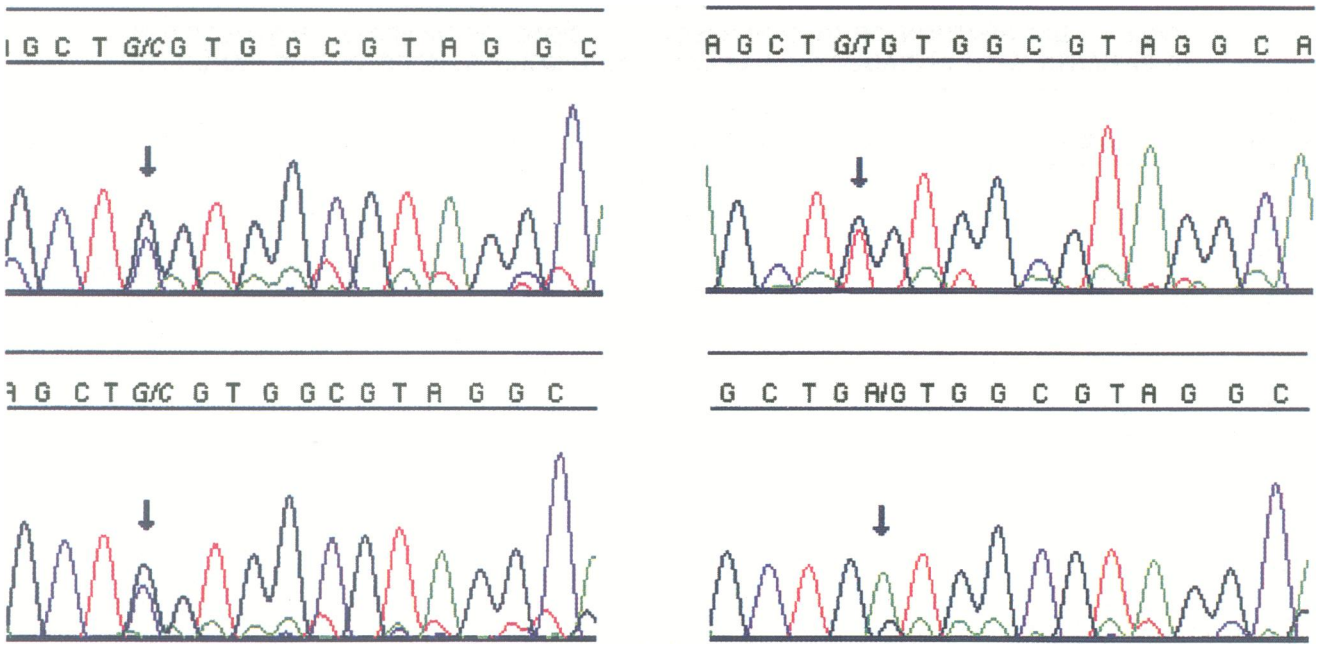

Figure 2. Representative results of DNA sequence analysis for K-ras gene exon 1. All sequences show the K-ras gene code from codon 11 (GCT) to codon 15 (GGC). Wild-type sequence is GCT GGT GGC GTA GGC (codon 12 GGT). Fluorescence spectroscopy analysis shows mutations (O) in all sequences: (A) K-ras mutation (CGT) detected in normal ductal epithelium (corresponding to patient 15, Table 1, and Fig. 1A; above left). (B) K-ras mutation (TGT) detected in a low-grade dysplasia lesion (patient 7, Table 1, and Fig. 1B; above right). (C) K-ras mutation (CGT) detected in a high-grade dysplasia–carcinoma *in situ* lesion (patient 8, Table 1, below left). (D) K-ras mutation (GAT) detected in a high-grade dysplasia–carcinoma *in situ* lesion (patient 4, Table 1, below right).

resection.[1–4,15] The molecular changes occurring in IPMT, a relatively uncommon tumor, have not been well documented and have not been analyzed previously regarding the stage or degree of neoplasia in a specific locus. The relevance of specific gene mutations to carcinogenesis in these tumors remains unknown.

The mean incidence of activating mutations in the K-ras gene of IPMT reported in the literature is 47.1% in 70 different cases examined in 6 studies,[29,35–39] but was 86% in a recent study of DNA from 7 patients evaluated by PCR and restriction fragment length polymorphism analysis.[35] Using PCR followed by automated sequencing with the dye terminator method in our patients with IPMT, we found activating codon 12 K-ras exon 1 mutations in 57% of the cancers. When multiple specimens were examined, a codon 12 mutation was found in 81.2% of patients. In our experience, this latter method was more accurate than restriction fragment length polymorphism in a previous study that detected K-ras mutations in 18% of papillary hyperplasias in chronic pancreatitis.[42] Reproducible objective readings have been more difficult to obtain with the restriction enzyme technique, and we have encountered problems with false-positive determinations. The prevalence of K-ras mutation was 66% in resected specimens from nine patients with pancreatic ductal adenocarcinoma, which were included as a comparison group. This prevalence of K-ras mutations in the adenocarcinomas is lower than in some reports[32] but agrees closely with the general experience.[19,21,24–34] Our results suggest that K-ras mutations are observed as frequently in patients with IPMT as in patients with ductal adenocarcinoma of the pancreas. Furthermore, the spectrum of mutations found in our study is the same as those found in other cancers and specifically in pancreatic ductal adenocarcinoma.[18,32] Our analysis, however, did show a relatively high number of GGT-to-CGT mutations and in one patient a mutation of codon 12, leading to a replacement of glycine by cysteine (TGT). The latter has not been described previously in IPMT[37–39] and is a relatively rare mutation in ductal pancreatic adenocarcinomas.

The analysis of the frequency of activating K-ras gene mutations along the spectrum of histologic lesions in IPMT showed a significant, stepwise increase as the degree of abnormality increased from normal duct–hyperplasia to the low-grade dysplasia, and then to CIS or invasive carcinoma. This finding is consistent with the hypothesis that activating K-ras gene mutations play a facilitating role in the transformation from normal epithe-

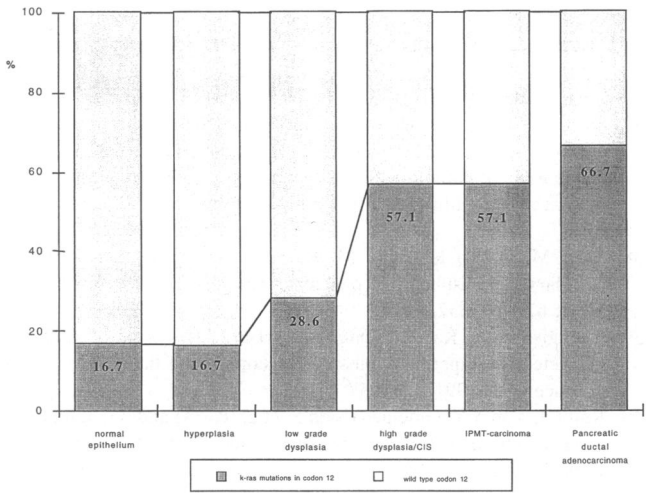

Figure 3. Analysis of the prevalence of K-ras gene mutations in codon 12 in different groups of histologic lesions as described in the Materials and Methods section. The statistical analysis shows that the stepwise increase in the frequency of K-ras mutations from normal ductal epithelium to intraductal papillary-mucinous carcinoma is significant ($p = 0.0168$).

lium to carcinoma in the majority of patients with IPMT. The similar frequencies of K-ras gene mutations in CIS and invasive carcinoma may imply that progression from an intraepithelial lesion to an invasive tumor may depend on factors other than K-ras mutations. Potentially involved in this process are mutations leading to p53 inactivation, c-erB-2 overexpression, and loss of Rb-gene product expression.[35,37,43] Mutations of the p53 tumor suppressor gene with subsequent detectable overexpression of the p53 protein have been shown to correlate with the grade of malignancy in IPMT.[35,37] A recent immunohistochemical study, however, did not detect p53 overexpression in nine cases of IPMT,[43] and Rivera et al.[42] and Hoshi et al.[44] could not detect p53 gene mutations by PCR and constant denaturant gel electrophoresis in 16 cases. Other oncogene changes, such as c-erB-2 overexpression or loss of expression of the Rb-gene product, have been shown, but a correlation with the degree of histopathologic abnormality has not been reported so far.[37,43]

We were interested to find a heterogeneous activating K-ras gene mutation of codon 12 in two specimens of histologically normal epithelium. The papillary hyperplasia and the low-grade dysplasia specimen microdissected on the same slides displayed identical mutations. Although this may be the first description of a K-ras mutation in histologically normal ductal epithelium,[36] our finding suggests that mutation of the K-ras gene may be a rather early event in mutagenesis. This also could imply that other parts of normal ductal epithelium, not resected during the operation, could harbor the mutation and therefore have an increased tendency toward neoplasia. Periodic imaging for surveillance of the residual pancreas seems advisable in these circumstances. However, because the two normal ducts were closer than 1 cm to histologically abnormal areas that also contained K-ras mutations, the finding must be considered with caution because of the theoretical possibility of contamination.

Our study shows a prevalence of activating mutations in codon 12 of the K-ras gene, which is as high in IPMT as in pancreatic ductal adenocarcinoma. The site-specific prevalence clearly increases with the degree of histopathologic abnormality in IPMT. We therefore conclude that K-ras gene mutations are important events in carcinogenesis in the majority of IPMT.

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Discussion

DR. JOHN L. CAMERON (Baltimore, Maryland): I enjoyed this paper nicely presented by Dr. Z'graggen, and I think it represents the type of work that we are going to be seeing presented at this Association more and more in the future, and less and less on how to manage the various pancreatic tumors surgically.

As we heard earlier in the meeting, there are good operations for pancreatic tumors that can be performed on virtually all patients in every age group, and I don't think we are going to see much more in the way of progress in the operative management of these tumors.

The problem is early detection. At least two thirds of our patients with pancreatic adenocarcinoma have metastatic tumor in regional nodes at the time of resection. What we need is a means of detecting these tumors before they have spread to regional nodes.

Six or eight years ago there was only one solid tumor about which anything was known concerning the molecular events that preceded the development of malignancy. That was from work done by Dr. Bert Vogelstein at Johns Hopkins demonstrating so beautifully the multiple molecular events that accompany the changes from normal colonic mucosa, to polyp formation, to high-grade dysplasia, carcinoma *in situ,* and then in invasive cancer. At that time, 6 or 8 years ago, there was virtually nothing known about the molecular events that accompany the transformation of ductal epithelium in the pancreas to malignancy.

But that has changed. In the course of just 6 or 8 years