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Diagnostic and Prognostic Value of Incidence of K-*ras* Codon 12 Mutations in Resected Distal Bile Duct Carcinoma

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Background and Objectives: The K-*ras* gene is one of the most extensively investigated oncogenes in a wide variety of human tumors, but has rarely been studied in distal bile duct carcinoma (DBDC). We sought to investigate the diagnostic and prognostic value of K-*ras* codon 12 mutations in this type of tumor.

Methods: Forty-seven patients who had undergone resection for DBDC were analyzed to reveal the incidence of K-*ras* codon 12 mutations, the locus most frequently involved. A rapid and simple two-step, semi-nested polymerase chain reaction (PCR) technique was used to detect mutations in paraffin-embedded tumor samples.

Results: The PCR mismatch amplification technique demonstrated that 35 (75%) of the 47 tumors harbored a point mutation in codon 12 of the K-*ras* oncogene. Patients with mutated tumors had no statistically different survival time compared to those patients without a mutation in the tumor. In contrast, negative microscopic margins proved to be a significant prognosticator.

Conclusions: K-*ras* codon 12 mutations are common in DBDC and may be useful in the diagnosis and early detection of these tumors. However, no prognostic value of these mutations could be identified in this analysis. The results of this study also suggest that negative surgical margins remain the mainstay of prognostication in resectable DBDC. However, due to the small number of patients included in this study, the results obtained should be interpreted with care. *J. Surg. Oncol. 1998;68:187–192.*

KEY WORDS: **oncogenes; K-*ras;* polymerase chain reaction (PCR); biliary surgery; biliary cancer**

INTRODUCTION

Malignant tumors arising from the distal bile duct (DBDC) are uncommon and by definition restricted to the intrapancreatic portion of the duct [1,2]. Spread along the ductal system to regional nodes, perineural invasion as well as hematogenous dissemination too often precludes curative resection, leaving palliative treatment as the only feasible option.

Even though the tumor stage of the resected specimen is an important prognostic parameter, its value as a prognosticator appears to be rather limited. The survival of the patients suffering from this malignancy might be improved if new prognostic markers could be defined which would determine those patients who would benefit from additional radiotherapy or chemotherapy.

The most promising new prognostic markers have resulted from cancer cell genetics. The role played by on-

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cogenes in the initiation of cancer or its progression has gradually come into focus, and one of the most extensively investigated is the family of *ras* oncogenes. Although the precise physiological function of *ras*-encoded proteins remains to be elucidated, an inherited predisposition towards developing cancer results from activating mutations at codons 12, 13, or 61. K-*ras* is the most common mutated oncogene and has been intensively investigated in pancreatic cancer, showing an overall frequency of 75% to 100% [3–7]. These data suggest that K-*ras* oncogene mutational activation is a critical event in the oncogenesis of most cancers arising from the exocrine pancreas.

The concept of examining the presence of K-*ras* oncogene in distal bile duct cancer is particularly attractive, since the pancreas and the bile duct are anatomically and embryonically related—both having developed from the end portion of the foregut. Thus far, only limited data are available precluding a systematic correlation between the incidence of K-*ras* mutations and prognosis. In addition, at the time of diagnosis it is often difficult to decide whether the tumor originates from the distal bile duct or from the pancreatic duct. It has been suggested that K-*ras* might be useful in differentiating these tumors, because a low frequency of K-*ras* mutations have been found in extrahepatic bile duct carcinomas [8,9].

The purpose of this study was to address the incidence of mutations in codon 12 of the K-*ras* oncogene and to investigate its prognostic and diagnostic value in resected DBDC.

MATERIALS AND METHODS

Patients and Tumor Histopathology

Three criteria were established for inclusion in this study. First, the patient had undergone subtotal pancreatoduodenectomy (Whipple procedure) with curative intention at the Academic Medical Center, Amsterdam. Second, the tumor had to be located in the intrapancreatic portion of the duct [1,2]. With large tumors, the main bulk of the tumor determined the site of origin. Third, there had to be evidence of a malignant histology. All the medical records and original histological paraffin-embedded tumor material from patients who underwent subtotal pancreatoduodenectomy between 1985 and 1996 were obtained and reviewed. Forty-seven patients fulfilled these criteria. Of these patients, 37 were male and 10 female, with a mean age of 60 years (range 37 to 77 years). Histopathological grading of the tumors was reviewed by one pathologist only (GJAO), and according to the WHO histological classification [10], subdivided into three categories: well, moderately, and poorly differentiated.

DNA Preparation

Paraffin-embedded specimens of DBDCs were cut into 4-μm sections and mounted on glass slides. Adjacent sections were stained with hematoxylin and eosin to confirm the presence of carcinoma tissue. The pathologist selected cell-rich areas of the tumor which were microdissected to minimize admixture with DNA from non-neoplastic tissue such as stromal and inflammatory cells. The tumor cells were collected in microcentrifuge tubes and incubated at 56°C overnight with 50–200-μl DNA isolation buffer (50 mmol/L TRIS-HCL, pH 8.5, 1 mmol/L ethylenediaminetetraacetic acid, and 0.2% Tween® 20) containing proteinase K (100 μg/ml). Proteinase K was inactivated for 10 min at 95°C. Of the resulting specimens, an aliquot of 1 μl was used for K-*ras* analysis.

Detection of K-*ras* Codon 12 Point Mutations

A two-step, semi-nested polymerase chain reaction (PCR) technique was used to detect mutations [11]. The advantage of this procedure is that it is possible to use paraffin-embedded material as a reliable source of DNA. In this technique, the K-*ras* codon 12 region is amplified using a mismatched primer which introduces an enzyme restriction site in the PCR products derived from wild-type K-*ras* alleles. In contrast, a restriction site is not generated if a mutation is present in codon 12 of K-*ras.* The PCR products are then incubated with the restriction enzyme. The PCR products with a mutation K-*ras* codon 12 sequence remain undigested and are solely amplified in the second PCR, yielding a mutant-enriched PCR product. Thereafter, the single-stranded PCR products are bound to nylon membranes and visualized by hybridizing with wild-type specific and mutant specific radioactive-labelled oligonucleotides followed by autoradiography (Fig. 1). Water is included as a control for contamination; specific synthetic oligonucleotides are added as controls for the hybridization. Amplification and digestion is controlled for by comparison of the dot blots pre- and postdigestion.

All analyses of the DNA samples were performed in duplicate to exclude technical artifacts. Autoradiograms were evaluated by three independent investigators without knowledge of the other features of these patients.

Statistical Methods

Kaplan-Meier survival analysis was used to estimate survival time, and univariate survival comparisons were made using the log-rank test (SPSS statistical software). $P < 0.05$ was considered statistically significant.

RESULTS

Among the 47 patients with DBDC in this study, three patients died after surgery (hospital mortality, 6%) due to septic complications and were not included in the survival study, leaving 44 cases in which follow-up information was complete. One patient was lost to follow-up after 2 years and was treated as a censored event at that

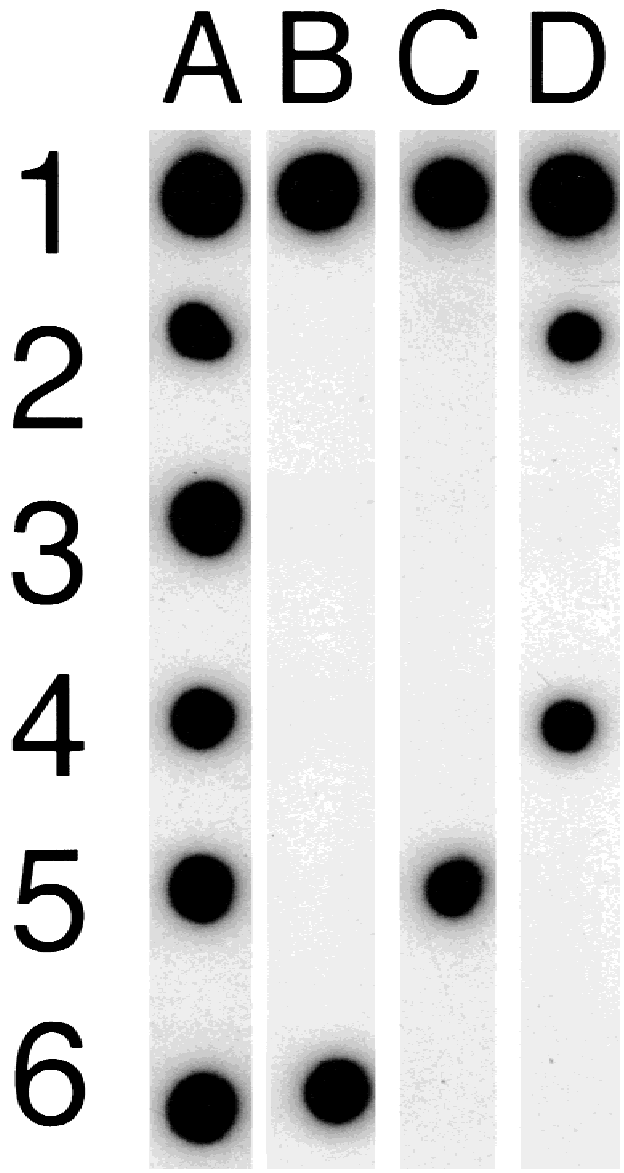

Fig. 1. Example of K-*ras* mutational analysis. Mutant-enriched PCR products are spot-blotted on separate nylon membranes which are hybridized with radioactive labeled oligonucleotides. Probe A was intended to detect the wild-type codon 12 sequence coding for glycine. The other probes were specific for three different K-*ras* codon 12 point mutations, glycine to arginine (B), glycine to valine (C), and glycine to aspartic acid (D). Row 1 is a positive control for hybridization. In patients 2, 4, 5, and 6, a point mutation of K-*ras* codon 12 was detected; a mutation was not detected in patient 3.

time. The medical records or autopsies revealed that the cause of death in all patients who died during the follow-up period was recurrent tumor. In addition to surgical resection, two patients received external beam radiation therapy, and one patient received both chemotherapy and radiation therapy. The median survival of patients with a resected DBDC was 20 months. A review of the histological slides revealed adenocarcinoma in all patients, of which one had a multifocal papillary adenocarcinoma. The majority of the patients (83%, 39/47) had a moderately or well-differentiated tumor. Negative microscopic margins were achieved in 25 (53%) patients.

TABLE I. Characteristics and Incidence of K-*ras* Mutations of the 47 Patients With Distal Bile Duct Carcinoma

	No. of samples (n = 47)	Mutant K-*ras* 35 (75%)
Sex		
Male	37	27 (73%)
Female	10	8 (80%)
Tumor differentiation		
Well-differentiated	9	6 (67%)
Moderately differentiated	30	23 (77%)
Poorly differentiated	8	6 (75%)
Lymph nodes[a]		
Involvement	21	16 (76%)
No involvement	26	19 (73%)
Vasoinvasive growth		
Present	19	15 (79%)
Absent	28	20 (71%)
Perineural invasion		
Present	29	23 (79%)
Absent	18	12 (67%)
Surgical margins		
Negative	25	16 (64%)
Positive	22	19 (86%)
Alive at 5-year follow-up[b]	7	4 (57%)

[a]The mean number of lymph node samples per tumor was 10.
[b]From 35 patients, the 5-year survival could be obtained.

Thirty-five (75%) of the 47 tumors harbored a point mutation in codon 12 of the K-*ras* oncogene (Table I). A number of potential prognostic factors were analyzed in a univariate model (Table II). K-*ras* codon 12 mutation was not significantly associated with decreased survival time ($P = 0.34$). Also, no correlation with K-*ras* mutations was found after stratification for negative surgical margins. If a negative microscopic margin was obtained after resection, survival was significantly prolonged ($P = 0.005$) (Fig. 2). Negative microscopic margins increased 5-year survival from 0% to 39% (7/18 patients with negative surgical margins and follow-up time of more than 5 years), and median survival from 13 months to 29 months. Moreover, tumor differentiation and perineural invasion were found to approach statistical significance with regard to survival. No significant differences in prognosis were found to be related to sex, status of lymph nodes, tumor size, or vasoinvasive growth.

Analysis of specific nucleotide changes at codon 12 revealed the occurrence of various mutations. The G to A transition at the second nucleotide of a codon 12, which converts the wild-type amino acid glycine (GGT) to aspartic acid (GAT), was the most prevalent mutation (Table III). Double mutations were not detected in these tumors. Duplicates were congruent in all cases.

DISCUSSION

In this study, the PCR demonstrated K-*ras* mutations in 75% (35/47) of patients who had undergone a resec-

TABLE II. Factors Influencing Survival After Resection for Distal Bile Duct Carcinoma

	Median survival (months)	*P* value
K-*ras* mutation		
Present	20	$P = 0.34$
Absent	23	
Sex		
Male	20	$P = 0.42$
Female	29	
Tumor size		
⩽2 cm	38	$P = 0.16$
>2 cm	13	
Tumor differentiation		
Well-differentiated	38	$P = 0.07$
Moderately differentiated	23	
Poorly differentiated	12	
Lymph nodes		
Involvement	23	$P = 0.11$
No involvement	13	
Vasoinvasive growth		
Present	13	$P = 0.10$
Absent	27	
Perineural invasion		
Present	13	$P = 0.05$
Absent	31	
Surgical margins		
Negative	29	$P = 0.005$
Positive	13	

tion for DBDC. Because of the low incidence of this type of tumor, comparable studies are limited and were performed on only a small number of patients. Motojima et al. [8] found seven (41%) K-*ras* codon 12 mutations in 17 patients and Caldas et al. [12] detected two mutations in codon 12 of K-*ras* in three patients with DBDC. Satoh et al. [13], in contrast, found no mutation at codon 12 in five patients with DBDC. It has been shown that extrahepatic bile duct carcinomas contain K-*ras* mutations in only a minority of the cells [14]. For this reason, besides the small study groups, variation in the sensitivity of the methods used and intratumor heterogeneity may account for the discrepancy in these studies.

This is the first report dealing with the relationship between K-*ras* mutation at codon 12 and prognosis in resected DBDC. Our results demonstrate that K-*ras* mutation does not appear to be an important prognostic factor in this type of malignancy. However, it should be emphasized that statistical analyses of the patient population in this study has restrictions due to the limited number of patients, precluding definite conclusions. K-*ras* point mutation has been reported to be the single most important prognostic factor in 69 patients with curative resected adenocarcinoma of the lung [15]. This observation with respect to lung carcinoma is supported by others [16]. However, in 75 patients with resected pancreatic carcinoma, no relationship could be found between point mutation in K-*ras* codon 12 and survival [3].

Negative surgical margins were the only independent prognostic determinant. In a previous study [17] from our department dealing with patients with DBDC who underwent subtotal pancreatoduodenectomy between 1983 and 1992, tumor size of more than 2 cm, lymph node involvement, and poor differentiation grade were also negative prognostic factors. These differences in results are attributive to the different criteria for selection in the two studies.

Because DBDCs have a higher overall 5-year survival rate than adenocarcinomas of the head of the pancreas, it is mandatory to distinguish clearly between these tumors. Since we detected a high incidence of K-*ras* oncogene activation in DBDC, we do not agree with others [8,9] that this molecular biological approach can be used diagnostically to differentiate between DBDC and pancreas carcinoma. Also, the spectrum of K-*ras* mutations in these tumors is often similar. Although several other mutations were found in this study, 51% (18/35) of the mutations were transitions at codon 12 that change glycine (GGT) to aspartic acid (GAT). These mutations were previously reported to be most prevalent in pancreatic carcinomas [3,5,9,18]. For these reasons both DBDC and pancreas carcinoma should be classified clinically according to their dominant histologic pattern and their location [19]. Point mutation of codon 12 in K-*ras*, on the other hand, could be a valuable diagnostic marker for the presence of carcinoma in the distal bile duct, especially in cases in which differentiation between benign inflammatory conditions and malignancy is difficult. Since the mutation occurs almost exclusively at codon 12, this can be done in a relatively simple molecular way without the need for numerous primers. It also enables an enrichment step that makes the technique sensitive and feasible on small samples. In addition, the glycine to aspartic acid substitution at codon 12 of K-*ras* oncogene is probably a specific target or has a higher potential to induce proliferation in DBDC and pancreatic carcinoma. In view of the similarities in mutation spectra, it is very suggestive that the environmental agent responsible for the induction of some mutations in pancreas carcinoma (e.g., cigarette smoking) is the same for DBDC; in fact, this type of mutation supports this notion.

Most studies concentrate on the extrahepatic biliary system as a uniform whole, and the reported incidence of K-*ras* mutations varies strongly in the literature [5,9,14,18–21]. Caution must be used in interpreting the results of these studies because, in our opinion, it is important to differentiate between proximal and distal bile duct carcinoma for a number of reasons. Firstly, the average 5-year survival rates for resected proximal and distal bile duct carcinomas are different, 14% [22,23] and 28% [17,22–24], respectively, suggesting a difference in biological behavior. Secondly, the surgical approach and type of resection differs—proximal tumors being usually

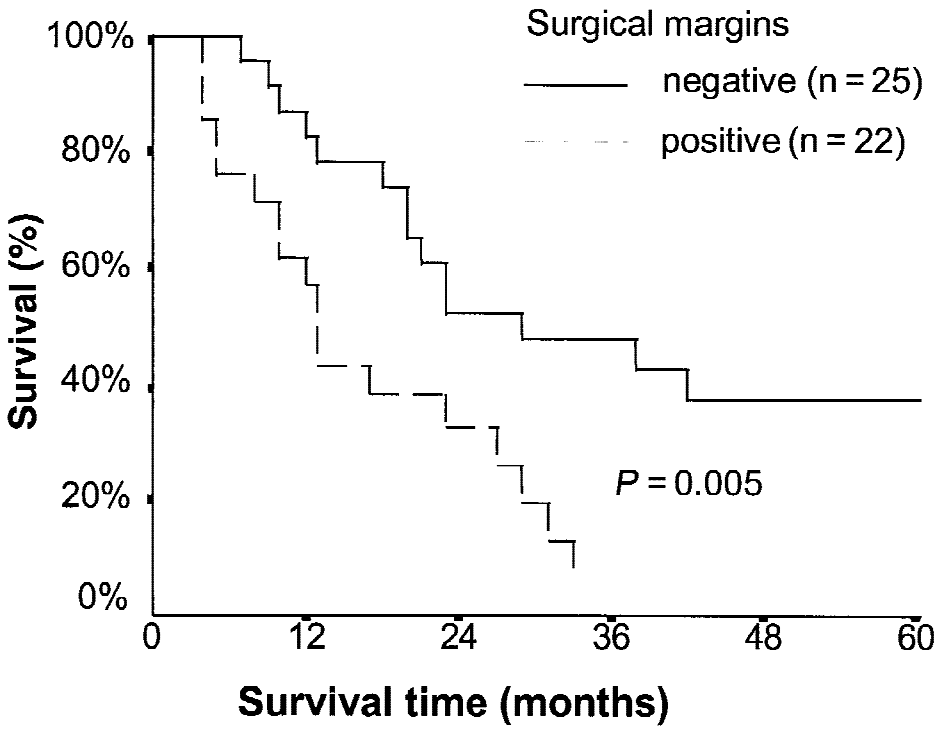

Fig. 2. Cumulative survival (Kaplan Meier curves) after subtotal pancreatoduodenectomy for the subgroups of resections with negative and positive surgical margins.

TABLE III. Spectrum of K-*ras* Mutations at Codon 12 in 35/47 Patients With Distal Bile Duct Carcinoma

Mutation	Amino acid	Incidence
G to C (CGT)	Arginine	6/35 (17%)
G to T (GTT)	Valine	11/35 (31.4%)
G to A (GAT)	Aspartic acid	18/35 (51.4%)

amenable to local resection. Finally, in this study the origin of the tumor was in each case established during preoperative evaluation and was defined by the strict topographical criteria described above. In large tumors this may be particularly difficult, since the location of the main bulk of the tumor occupying the pancreatic head does not reveal the site of origin. For this reason, pancreatic cancer may be misdiagnosed as DBDC, with a consequent increase of the ratio mutant/normal *ras* gene. Because 92% (36/39) of the tumors in this series were less than 3 cm in size, the influence of this potential diagnostic error is estimated to be low in our study.

The precise mechanism whereby the mutated *ras* gene disrupts normal growth control mechanisms is unclear, but they act in a fashion dominant to the wild-type (normal) allele [25]. The finding that a significant proportion of the tumors in this study did harbor a K-*ras* codon 12 mutation indicates that activation of this oncogene must be an important event in distal biliary tract carcinogenesis. Since 25.5% (12/47) of DBDC contained no mutation, it is possible that a minority of these tumors may develop through a different pathway of genetic alterations which does not involve *ras* mutational activation. K-*ras* mutations have also been detected in adenomas and hyperplastic lesions in different tissues [7,20,26,27], but it seems unlikely that all these mutated lesions develop into carcinoma [7]. Therefore, in agreement with other investigators [14,20,28], we believe that K-*ras* is an important early event in carcinogenesis of DBDC and is probably not solely responsible for malignant transformation, but merely part of multiple genetic lesions.

In conclusion, although K-*ras* codon 12 mutations were very frequently found in DBDC (75%, 35/47), no prognostic value of these mutations could be identified in this analysis. It may prove useful in the diagnosis and early detection of these tumors, but this should be studied in a series also containing patients with other conditions to judge specificity. Because of the limited number of patients studied, the results of this analysis must be viewed with caution. It is, however, clear that differentiation of DBDC from other periampullary carcinomas cannot be based on K-*ras* mutation detection alone, and that the state of the surgical margins remains the mainstay of prognostication in patients treated with intentionally, curative surgery.

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