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CANCER

Clinical validity of detecting K-*ras* mutations for the diagnosis of exocrine pancreatic cancer: a prospective study in a clinically-relevant spectrum of patients

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Abstract The diagnostic utility of detecting K-*ras* mutations for the diagnosis of exocrine pancreatic cancer (EPC) has not been properly studied, and few reports have analysed a clinically relevant spectrum of patients. The objective was to evaluate the clinical validity of detecting K-*ras* mutations in the diagnosis of EPC in a large sample of clinically relevant patients. We prospectively identified 374 patients in whom one of the following diagnoses was suspected at hospital admission: EPC, chronic pancreatitis, pancreatic cysts, and cancer of the extrahepatic biliary system. Mutations in the K-*ras* oncogene were analysed by PCR and artificial RFLP in 212 patients. The sensitivity and specificity of the K-*ras* mutational status for the diagnosis of EPC were 77.7% (95% CI: 69.2–84.8) and 78.0% (68.1–86.0), respectively. The diagnostic accuracy was hardly modified by sex and age. In patients with either mutated K-*ras* or CEA > 5 ng/ml, the sensitivity and specificity were 81.0% (72.9–87.6) and 62.6% (72.9–87.6), respectively. In patients with mutated K-*ras* and CEA > 5 ng/ml the sensitivity was markedly reduced. In comparisons with a variety of non-EPC patient groups sensitivity and specificity were both always greater than 75%. In this clinically relevant sample of patients the sensitivity and specificity of K-*ras* mutations were not sufficiently high for independent diagnostic use. However, it seems premature to rule out the utility of K-*ras* analysis in conjunction with other genetic and 'omics' technologies.

Keywords KRAS oncogene · Genes, ras · Oncogene protein p21 ras · Pancreatic neoplasms/diagnosis · Research/methods · Diagnosis/diagnostic use · Diagnosis/analysis · Diagnosis/methods · Epidemiology/diagnosis

For the PANKRAS II Study Group: Members of the Multicentre Prospective Study on the Role of the K-*ras* and other Genetic Alterations in the Diagnosis, Prognosis and Etiology of Pancreatic and Biliary Diseases (PANKRAS II) Study Group are mentioned in previous publications.

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Abbreviations

BPD	Benign pancreatic disease
CEBS	Cancer of the extra-hepatic biliary system
CI	Confidence interval
CP	Chronic pancreatitis
EPC	Exocrine pancreatic cancer
ERCP	Endoscopic retrograde cholangio-pancreatography
IQR	Interquartile range
OBD	Other benign disease
OM	Other malignancy
OR	Odds ratio

Introduction

The diagnosis of exocrine pancreatic cancer (EPC) remains a difficult challenge in clinical care. Early symptoms and laboratory findings are usually non-specific [1–4], and few tumour markers are widely accepted for routine use [2, 3, 5]. When pancreatic cancer is clinically suspected, imaging techniques offer good diagnostic efficiency. Computed tomography (CT), the most sensitive procedure, non-invasively identifies approximately 90% of cases [6]. However, since CT has less than perfect specificity (approximately 54%), cytological confirmation of radiology findings is usually required. The only curative treatment for EPC is surgical resection, but it is feasible in as few as 20% of cases [1, 2], and is not without problems [7–11]. More rapid and accurate methods to diagnose EPC are hence vital to identify patients who would benefit from surgical resection.

Activating point mutations at codon 12 of the K-*ras* gene are the most frequent oncogene alteration in EPC; at diagnosis they are detected in 75–90% of patients with EPC [12, 13]. Therefore, it has long been argued that detecting K-*ras* mutations could be clinically useful in the differential diagnosis of EPC and related disorders [14]. In blood or stools of pancreatic patients K-*ras* mutations are detected at a much lower frequency than in tumour tissue [14–16]; thus, detection in such samples has not been proven effective in EPC diagnosis. Similarly, detecting mutated K-*ras* in pancreatic juice or bile has limited diagnostic usefulness [17]. By contrast, K-*ras* analysis can feasibly be performed in cytohistological specimens, which are often obtained through pancreatic duct brushing, fine needle aspiration, percutaneous biopsy or at surgery. Whether detecting K-*ras* mutations in these specimens has diagnostic utility is not established, with few reports of sufficient methodological quality, and conflicting results [14]: studies recommend detecting mutated K-*ras* for diagnosis of EPC as an independent test [18, 19], as an adjunct to cytology [20, 21], only in certain situations such as atypical cytology [22, 23], or not at all [7, 24, 25].

The diagnostic validity of a potential test should be evaluated in relevant populations [26–30]. It is particularly important that the ultimate stage of validation be carried out in a population as close as possible to that in which the test would be used in practice. Therefore, reports demonstrating the utility of K-*ras* mutations for distinguishing between patients diagnosed with pancreatic cancer and a group of controls (either healthy or with a distinct diagnosis) do not reflect the relevant clinical practice, where the test would be used to detect EPC among symptomatic patients with apparently related and competing diagnoses. Furthermore, to allow for biological variation, it is important to assess the test in a large number patients. None of the above mentioned studies on K-*ras* and EPC meets these requirements: most studies had a limited patient spectrum, reduced study size, or inadequate description of inclusion criteria.

The aim of this study was to evaluate the clinical validity of detecting K-*ras* mutations in the diagnosis of EPC in a sample of clinically relevant patients.

Patients and methods

Selection of patients

Detailed methods of the PANKRAS II study have been previously described [4, 31–43]. Briefly, subject recruitment took place between 1992 and 1995 at five general hospitals in the eastern Mediterranean part of Spain. Aided by the study research physicians, trained monitors prospectively identified hospital patients in whom one of the following diagnoses was suspected at admission: cancer of the exocrine pancreas, chronic pancreatitis, pancreatic cysts and pseudocysts (including those secondary to acute pancreatitis), and cancer of the extrahepatic biliary system [32, 33]. The broad eligibility criteria are due to the main objective of the PANKRAS II study: to assess the clinical usefulness of detecting mutations in the K-*ras* for the diagnosis of cancers of the exocrine pancreas and the

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extrahepatic biliary system. A total of 602 subjects were selected. The ethics committees of the participating hospitals approved the study protocol, and all patients gave consent to be included in the study.

The present report is based on patients in whom a diagnosis of EPC was suspected at hospital intake, or who were definitively diagnosed with EPC during the study. Furthermore, the analysis is limited to individuals for whom cytological or histological material was available for K-*ras* analysis and in whom mutational status was determined (Fig. 1). Table 1 presents the socio-demographic and clinical characteristics of the study population and compares the final study population with the individuals who met the inclusion criteria but in whom K-*ras* mutational status was not established [44].

Data collection

A structured form was used to collect clinico-pathological information from medical records, including details on presenting symptoms, past medical history, the physical examination at admission, ancillary diagnostic procedures, laboratory results and follow-up care [32, 33, 41, 45]. Diagnostic tests included ultrasound scan, computerized axial tomography, fibrogastroscopy, endoscopic retrograde cholangio-pancreatography (ERCP), transparietohepatic cholangiography (TPHC), gammagraphy, laparoscopy and exploratory laparotomy [46]. We classified as 'invasive' diagnostic tests fibrogastroscopy, ERCP, TPHC, gammagraphy, laparoscopy and laparotomy. When a diagnostic procedure was performed more than once, the physician who extracted the data chose the more informative result. Socio-demographic information and additional information regarding symptoms and medical history were obtained directly from patients during their hospital stay by interview with trained monitors [4, 44].

Definitive diagnosis

A panel of experts in gastrointestinal diseases, comprising surgeons and gastroenterologists with extensive experience in pancreatic and biliary cancer, a medical oncologist and several physician epidemiologists, reviewed the hospital discharge diagnosis and all pathological and clinical information available, including follow-up, to achieve a consensual definitive diagnosis [32, 33, 44] This process was carried out blinded to the results of K-*ras* analysis and has also been described elsewhere in detail [33]. The definitive diagnosis was categorised into five groups: EPC, cancer of the extra-hepatic biliary system, other

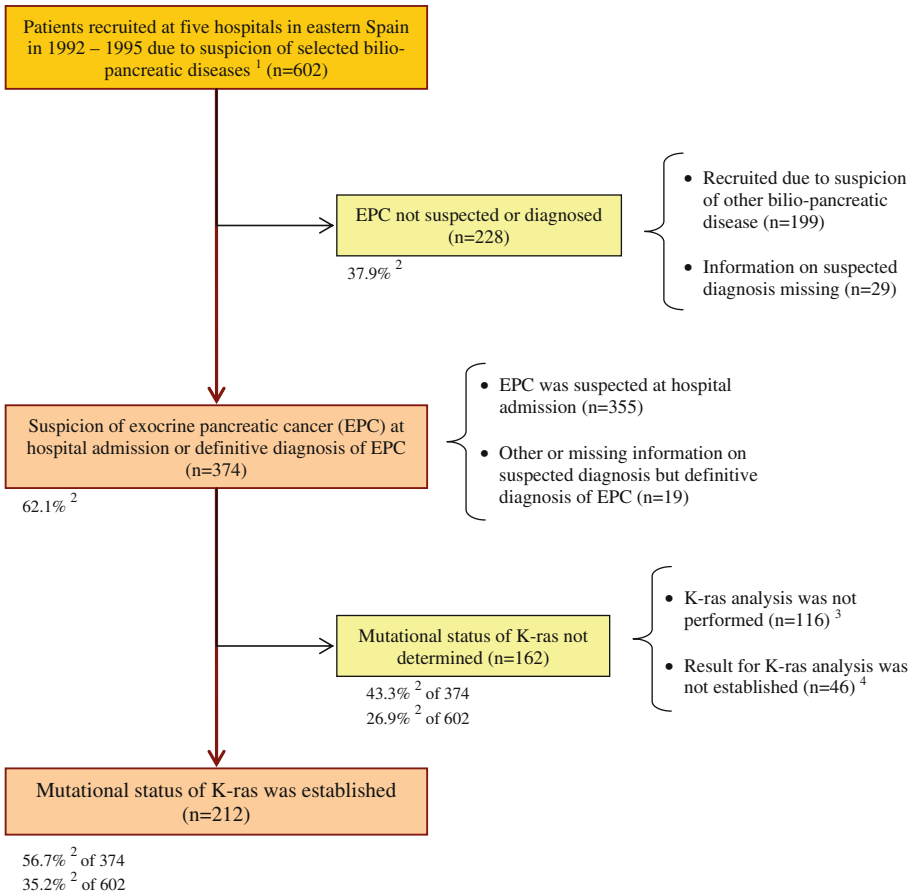

Fig. 1 Flow of participants through the recruitment process. *1* At least one of the following diagnoses was suspected at hospital admission: exocrine pancreatic cancer (EPC), chronic pancreatitis, pancreatic cysts and pseudocysts, cancer of the extra-hepatic biliary system. *2* Percentage calculated with respect to the box immediately above (e.g., 37.9% = 228/602 * 100). *3* 43 (37.1%) cases were definitively diagnosed as EPC. *4* 21 (45.7%) cases were definitively diagnosed as EPC

Table 1 Comparison of 212 patients included in analysis with those who met inclusion criteria but were excluded ($n = 162$) due to lack of the index test

	Total ($n = 374$) N (%)	K-*ras* determined		*P* value
		Yes ($n = 212$) *N* (%)	No ($n = 162$) *N* (%)	
Age				
Mean ± SD	66.6 ± 13.5	64.8 ± 13.3	68.9 ± 13.4	0.003[a]
Median	68.0	65.8	70.8	0.003[b]
Minimum	26.0	26.0	36.7	
Maximum	93.6	91.9	93.6	
Sex				
Male	221 (59.1)	121 (57.1)	100 (61.7)	0.364[c]
Female	153 (40.9)	91 (42.9)	62 (38.3)	
Definitive diagnosis				
Exocrine pancreatic cancer	185 (49.5)	121 (57.1)	64 (39.5)	
Cancer extra-hepatic biliary system	63 (16.8)	35 (16.5)	28 (17.3)	<0.001[c]
Benign pancreatic diseases	51 (13.6)	21 (9.9)	30 (18.5)	
Other benign diseases	39 (10.4)	10 (4.7)	29 (17.9)	
Other malignancies	36 (9.6)	25 (11.8)	11 (6.8)	
Diagnostic work-up				
Cytology	174 (46.6)	118 (55.7)	56 (34.8)	<0.001[c]
Histology	235 (62.8)	165 (77.8)	70 (43.2)	<0.001[c]
Ultrasound scan	342 (91.4)	195 (92.0)	147 (90.7)	0.671[c]
Fibrogastroscopy	124 (33.2)	69 (32.5)	55 (34.2)	0.743[c]
Computed tomography	290 (77.5)	169 (79.7)	121 (74.7)	0.248[c]
ERCP	163 (43.6)	80 (37.7)	83 (51.2)	0.009[c]
Exploratory laparotomy	198 (52.9)	137 (64.6)	61 (37.7)	<0.001[c]
Study hospitals				
Hospital general de Elche	67 (17.9)	45 (21.1)	22 (13.6)	0.004[c]
Hospital del Mar	68 (18.2)	37 (17.5)	31 (19.1)	
Hospital de Son Dureta	78 (20.9)	51 (24.1)	27 (16.7)	
Hospital Valld'Hebron	112 (29.9)	48 (22.6)	64 (39.5)	
Hospital Mutua de Terrassa	49 (13.1)	31 (14.6)	18 (11.1)	

[a] Student's *t* test (*two-tailed*)

[b] Kruskal–Wallis' test

[c] Pearson's chi-squared test (*two-tail*)

ERCP Endoscopic retrograde cholangio-pancreatography

malignancy, benign pancreatic disease, and other benign disease. For the purpose of this study, these groups were used for the reference standard. The diagnostic index was calculated comparing patients definitively diagnosed with EPC with all other patients in the remaining four categories.

Detection of K-*ras* mutations

Details of laboratory protocols have also been described elsewhere [31, 36–44]. Briefly, mutations in codon 12 of K-*ras* oncogene were studied using DNA extracted from paraffin-embedded tumor tissue. Amplifications were done in two steps by nested PCR; an artificial BstNI restriction endonuclease site was introduced to discriminate between wild-type and mutated K-*ras* codon 12 sequences. Products were analyzed by acrylamide gel electrophoresis and ethidium bromide staining. This technique was able to detect one homozygous mutated cell in the presence of 10^2 normal cells. To characterize the nucleotide substitution in codon 12, all mutated samples were further analyzed using a similar RFLP-based approach. Interpretation of digestion products' electrophoresis was performed independently by two investigators to confirm the results.

Statistical analysis

Univariate statistics were computed as customary [47]. For comparisons between continuous variables, Mann–Whitney's *U* test and Student's *t* test were used. In contingency

tables, Fisher's exact test for homogeneity or independence was applied to assess the relationship between two categorical variables. Diagnostic accuracy of detecting K-*ras* mutation was expressed in terms of sensitivity and specificity with their corresponding 95% confidence intervals (CI) [26, 28]. Data analysis was performed with SPSS 15.0 and Stata 8.0.

Results

Representativeness of the sample of patients

In this study, 212 patients contributed to the analysis of the diagnostic potential of detecting K-*ras* mutations. Although we prospectively attempted to include all individuals with suspected EPC or a definitive diagnosis of EPC, we were unable to include 162 patients due, first, to unavailability of a sample suitable for K-*ras* analysis and, to a lesser extent, to failure to establish the mutational status of the oncogene. Table 1 shows that excluded individuals were older than patients included in the analyses, and were less likely to be diagnosed definitively with EPC. Differences were observed in the diagnostic techniques performed in these two patient groups and in the recruitment hospital; diagnostic procedures vary by hospital and are linked to availability of tissue sample, the primary factor relating to failure to determine K-*ras*. Furthermore, the diagnostic work-up is influenced by a number of factors, including patient characteristics as age (e.g., older patients are less likely to undergo invasive tests).

Definitive diagnoses and K-*ras* mutations

Among patients included in the study, the most frequent pathologies were EPC ($n = 121$ patients, 57.1%) and cancer of the extrahepatic biliary system (35, 16.5%). K-*ras* codon 12 was mutated in approximately half of all patients (114, 53.8%). While K-*ras* mutations were observed in all diagnostic groups (Table 2), the frequency was highest in patients definitively diagnosed with EPC (94, 77.7%), followed by cancer of the extra-hepatic biliary system (11, 31.4%), and other malignant diseases (6, 24.0%). Thus, 27 of the 121 subjects with EPC (22.3%) had wild-type K-*ras*. Tumours of patients with EPC had the following stage distribution: 25 (20.7%) were in stage I, 21 (17.4%) in stage II, 15 (12.4%) in stage III, and 60 (48.6%) in stage IV. There was no significant difference in the prevalence of mutated K-*ras* across stages, with 18 (72%) of stage I cancers harbouring the mutation, 19 (90.5%) of stage II, 12 (80.0%) of stage III, and 45 (75%) of stage IV cancers ($P = 0.441$).

Diagnostic validity of K-*ras* mutations for exocrine pancreatic cancer

The sensitivity and specificity of the K-*ras* mutational status for the diagnosis of EPC in our patient sample were 77.7% (95% CI: 69.2–84.8) and 78.0% (68.1–86.0), respectively. If we limit the analysis to the 196 patients who entered the study with a suspected diagnosis of EPC, thus excluding 16 patients with a definitive diagnosis of EPC but missing information on the suspected diagnosis, the values of sensitivity and specificity were almost identical (77.1 and 78.0%, respectively). The diagnostic accuracy was unmodified by the sex or age of the patient (Table 3). We also evaluated the diagnostic validity of K-*ras* in certain clinical subgroups related with results from blood analyses and imaging tests, and found that the observed values for both sensitivity and specificity did not change significantly (Supplementary data, Tables S1, S2, and S3). We further assessed the potential clinical validity of detecting K-*ras* mutations in combination with other clinical information. Thus, in patients with either mutated K-*ras* or CEA > 5 ng/ml, the sensitivity and specificity were 81.0% (72.9–87.6) and 62.6% (72.9–87.6), respectively. When we considered only patients with mutated K-*ras* and CEA > 5 ng/ml the sensitivity was markedly

Table 2 Prevalence of K-*ras* mutations by definitive diagnosis

	Total ($n = 212$) *N (%)*	K-*ras*		*P* value[a]
		Mutated ($n = 114$) *N (%)*	Wild-type ($n = 98$) *N (%)*	
Definitive diagnosis				
Cancer of the exocrine pancreas	121 (57.1)	94 (82.5)	27 (27.6)	<0.001
Cancer of the extrahepatic biliary system	35 (16.5)	11 (9.6)	24 (24.5)	
Benign pancreatic diseases	21 (9.9)	1 (0.9)	20 (20.4)	
Other benign diseases	10 (4.7)	2 (1.8)	8 (8.2)	
Other malignancies	25 (11.8)	6 (5.3)	19 (19.4)	

[a] Pearson's chi-squared test

Table 3 Sensitivity and specificity of K-*ras* mutations for the diagnosis of exocrine pancreatic cancer (EPC), overall and stratified by age and sex

	Total	EPC ($n = 121$)		P^a	Sensitivity	Not EPC ($n = 91$)		P^a	Specificity
		K-*ras* mutated	K-*ras* wild type		% (95% CI)	K-*ras* mutated	K-*ras* wild type		% (95% CI)
Total	212	94	27		77.7 (69.2–84.8)	20	71		78.0 (68.1–86.0)
Sex									
Male	121	55	16	0.944	77.5 (66.0–86.5)	11	39	0.996	78.0 (64.0–88.5)
Female	91	39	11		78.0 (64.0–88.5)	9	32		78.0 (62.4–89.4)
Age									
≤65 years	102	46	16	0.344	74.2 (61.5–84.5)	9	31	0.915	77.5 (61.5–89.2)
>65 years	110	48	11		81.4 (69.1–90.3)	11	40		78.4 (64.7–88.7)

[a] Pearson's chi-squared test

reduced. This pattern was seen with all other analytes considered (Supplementary data, Tables S4 and S5). Results on the diagnostic accuracy of K-*ras* according to different comparisons and patient populations are shown in Table S6. Sensitivity was below 60% when the target disease was all malignant disorders, which is an uncommon clinical scenario. For all other comparisons, sensitivity and specificity were both greater than 75% (Supplementary data, Table S6).

Discussion

In this clinically relevant large sample of patients the sensitivity and specificity of K-*ras* mutations were less than ideal, 77.7% (95% CI: 69.2–84.8) and 78.0% (68.1–86.0), respectively. The clinical validity of mutated K-*ras* as an independent form of identifying EPC is hence limited, because more than 20% of the cases would erroneously be classified as non-pancreatic cancer (*false negatives*), and at least 20% of patients classified as EPC due to mutated K-*ras* would actually suffer from a distinct disease (*false positives*).

Diagnostic accuracy values remained remarkably stable when socio-demographic or clinical variables were considered. In this study, K-*ras* analysis was performed in histological and cytological samples, thus taking advantage of the two common procedures. K-*ras* mutational analysis could be used as an adjunct to cytology, potentially increasing the sensitivity, or it could be ordered only in cases where cytology is negative or inconclusive. In this study, we were unable to consider these scenarios, whose feasibility in a research study is uncertain.

As expected on clinical grounds, it was not possible to perform K-*ras* analysis in all patients who entered the PANKRAS II study with a suspicion of EPC. Nevertheless, selection criteria are among the widest on this field, and the pragmatic allowance of different methods of tissue collection made it possible to include a patient spectrum rather greater than in most previous studies [14]. The prompt identification of cases is an another strength of the study [48]. We included 19 patients who were definitively diagnosed with EPC during the study but in whom information regarding suspected diagnosis at hospital admission was missing. The decision to include these patients did not take into account their K-*ras* mutational status and, therefore, the diagnostic sensitivity we calculated was not influenced. In our population the prevalence of EPC may be different than in other clinical contexts; since positive and negative predictive values are highly influenced by disease prevalence, the predictive values of K-*ras* mutations will differ in other settings from values estimated in this report [27–30].

Although fieldwork for PANKRAS II study was carried out more than a decade ago, in our view the results presented here are pertinent and relevant today. During this time little progress has been made in the development of valid, evidence-based diagnostic procedures, including new molecular and 'omics' techniques [14]. Also regrettably, patients continue to be diagnosed late, often precluding any chance of curative treatment. On a population basis, survival rates for this aggressive tumour have remained stable in many regions worldwide [2, 49].

In many cases, cytological or histological samples were collected during exploratory laparotomy, a relatively common procedure when EPC is suspected. However, K-*ras* analysis was also common in cytological samples obtained through less invasive measures as fine needle aspiration. Nevertheless, the sensitivity and specificity of K-*ras* did not depend on the method of tissue collection.

In conclusion, we have calculated the sensitivity and specificity of K-*ras* mutation for detecting EPC in a wide spectrum of patients with a variety of related pathologies common in many clinical settings. While the diagnostic

index for detecting K-*ras* mutations in this context was not sufficiently high for independent diagnostic use, it seems premature to rule out the analysis of K-*ras* in conjunction with other technologies, especially in light of the advances in the 'omics' fields.

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