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# Prospective evaluation of the contribution of K-*ras* mutational analysis and CA 19.9 measurement to cytological diagnosis in patients with clinical suspicion of pancreatic cancer

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**Abstract**

The aim of this study was to prospectively evaluate the diagnostic contribution of the detection of K-*ras* mutation and measurement of serum CA 19.9 concentrations to cytological diagnosis in patients with clinical suspicion of pancreatic cancer. These patients had either the presence or absence of a pancreatic mass as determined by imaging procedures. A total of 156 consecutive patients with clinical suspicion of pancreatic cancer or for confirmation and follow-up of their chronic pancreatitis disease were included: 84 patients presenting a pancreatic mass (group 1) and 72 patients without a pancreatic mass (group 2). K-*ras* mutations were detected by a restriction fragment length polymorphism/polymerase chain reaction (RFLP/PCR) method and CA 19.9 by an immunoluminometric assay. When a pancreatic mass was present, cytology offered a high sensitivity, but with a significant number of inconclusive results and K-*ras* mutational analysis offered a highly specific test. In the absence of a pancreatic mass, CA 19.9 (cut-off 100 U/ml) increased the sensitivity of the diagnosis by cytology and K-*ras* mutational analysis did not add significant information. Thus both tests contribute to the clinical decision process when pancreatic cancer is clinically suspected and the cytological report is not conclusive. 

*Keywords:* CA 19.9; K-*ras*; Pancreatic neoplasm; Chronic pancreatitis; Cytology; Pancreatic juice; Fine needle aspirates (FNA)

## 1. Introduction

The incidence of pancreatic cancer has progressively increased over the last decades. Despite its poor prognosis, patients with localised disease may be cured with surgery; however, it is still difficult to diagnose pancreatic cancer at these earlier stages. When pancreatic cancer is clinically suspected endoscopic retrograde cholangiopancreatography (ERCP) may facilitate its early detection and allows the collection of pancreatic juice for cytological examination [1,2]. If a pancreatic mass is identified by ultrasonography or computed tomography (CT) scan, a guided percutaneous fine-needle aspirate (FNA) is performed in order to make the cytological diagnosis [3]. Due to the high number of cases without a conclusive diagnosis, it would be worth complementing cytology with other diagnostic tools.

The high incidence of mutations in codon 12 of the K-*ras* gene in pancreatic cancer (65–100%) [4] in association with a minor (less than 5%) incidence in codons 13 and 61 and its early appearance in pancreatic tumorigenesis [5] led to K-*ras* mutations being considered as a potential tumour marker at the tissue level. Data suggest that the detection of these mutations in specimens obtained by FNA or from pancreatic juice may improve the diagnostic accuracy of the cytological examination [6–11]. However, K-*ras* mutations have been detected, not only in intraductal carcinomas, but

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also in pancreatic mucinous cell hyperplasia [12,13] and chronic pancreatitis [14,15], a finding that is likely to limit its value in the diagnosis of pancreatic cancer.

CA 19.9 is the carbohydrate antigen defined by the monoclonal antibody 1116 NS 19.9 and is the sialylated Lewis[a] blood group antigen. Elevated serum CA 19.9 concentrations are found in a high proportion of patients with pancreatic cancer and CA19.9 is considered to be the standard serum marker in the management of adenocarcinoma of the pancreas [16]. However, CA 19.9 is often falsely elevated in benign pancreatic diseases, especially in chronic pancreatitis. In order to increase the specificity of CA 19.9 as a diagnostic tool, several authors have used cut-off values higher than the superior limit of the reference interval. Using concentrations equal to or higher than 100 U/ml, sensitivities obtained ranged from 55% and 89% with a specificity of approximately 90% [17]. Safi and colleagues [18] showed that at the time of diagnosis, a CA19.9 serum value above 120 U/ml was present in 77% of patients with pancreatic carcinoma. Kim and colleagues [19] in a recent publication proposed a cut-off value of 37 U/ml for differentiating pancreatic and biliary cancers from other benign diseases, but if patients show symptoms and signs of acute cholangitis or cholestasis a cut-off value of 300 U/ml was suggested. In our previous experience the optimum cut-off value obtained, based on receiver operating characteristics (ROC) curve analysis, when a pancreatic mass was present, proved to be 250 U/ml with a sensitivity and specificity of 60% and 90%, respectively, and without a mass, the cut-off value was 100 U/ml [20].

In the present study, we have prospectively evaluated the clinical utility of the detection of K-*ras* mutations and the serum CA 19.9 values in the diagnosis of pancreatic cancer. Patients have been grouped into two categories: (i) in the presence of a pancreatic mass where pancreatic cancer is more likely; and (ii) in the absence of a mass, mainly in the context of chronic pancreatitis. In both situations, much attention has been paid to the relative contribution of both markers when the cytological report is not conclusive due to the presence of suspicious cells, when cellular material was insufficient or when normal-appearing duct cells were reported.

## 2. Patients and methods

### 2.1. Patients and samples

Initially, 171 consecutive patients admitted to our hospital between January 1995 and December 1998 with clinical suspicion of pancreatic cancer were included. In 15 cases (9%) (11 with a pancreatic mass and 4 without a mass) incomplete clinical information and/or inadequate sample collection led to their exclusion. A final population of 156 patients was considered. Patients were classified into two different groups according to the presence or absence of a pancreatic mass.

Group 1 included 84 patients (47 men (56%) and 37 women (44%) mean age 66 years; range: 35–86) with clinical symptoms of jaundice, weight loss or abdominal pain, where the detection of a pancreatic mass was identified by means of imaging techniques. K-*ras* mutational analysis of 51 of the 84 (61%) patients has been reported elsewhere [21]. FNAs of the masses were percutaneously obtained under ultrasonography (90%) or CT (10%) guidance. Every specimen obtained from pancreatic FNA was divided into two parts: one part was immediately examined cytologically as fresh smears and the other part was analysed for the presence of K-*ras* mutations. Sera from these patients were obtained before the FNA procedure. The diagnosis of pancreatic carcinoma was established if malignant cells were identified in the FNA samples and/or when death occurred within the first year after diagnosis with clinical evolution compatible with disseminated cancer disease. Diagnosis of chronic pancreatitis was based on standard clinical criteria (including clinical symptoms, abnormal ultrasonography or CT scan findings, abnormal previous ERCP and impaired pancreatic exocrine function when assessed) or pathological confirmation if possible. Final diagnoses were 60 pancreatic adenocarcinomas, 2 mucinous cystic tumours, 4 endocrine tumours and 6 other malignancies (4 cholangiocarcinomas and 2 lung metastases). Benign diseases were as follows: 10 chronic pancreatitis, 1 acute pancreatitis and 1 tuberculosis (Table 1).

Group 2 included 72 patients (58 (81%) men and 14 (19%) women; mean age 56 years; range: 29–78) with no evidence of a pancreatic mass and clinical symptoms of a pancreatic disease: patients had chronic pancreatitis (see above described criteria) according to the findings of US or CT scan, abnormal previous ERCP, impaired pancreatic exocrine function when assessed, pancreatitis of unknown origin or obstructive jaundice without gallstones, in whom a diagnostic, control or therapeutic ERCP was indicated. K-*ras* mutational analysis of 30 of the 72 (42%) patients has been reported elsewhere [22]. The majority of them ($n = 51$; 71%) were chronic pancreatitis patients in whom an ERCP was indicated in order to confirm stage and/or follow-up of the disease. ERCP was performed using secretin stimulation to collect pancreatic juice samples. After collection, pancreatic juices were centrifuged and the resulting pellets examined cytologically and analysed for mutations in codon 12 of K-*ras*. Sera from these patients were obtained before the ERCP procedure. In this set of patients, a minimum of 12 months (range: 12–37) follow-up period was available. Routine clinical follow-up visits are being continued every 3–6 months; either imaging techniques or function tests are performed when clinically

Table 1
Incidence of K-*ras* mutations by RFLP/PCR methods and CA 19.9 determination according to cytological diagnosis in patients with a pancreatic mass ($n = 84$)

| Final diagnosis | Patients ($n$) (%) | | Cytology | | | |
|---|---|---|---|---|---|---|
| | | | Malignant cells | Suspicious cells | Insufficient material | Non-malignant cells |
| Malignant diseases | | | | | | |
| Pancreatic ductal adenocarcinoma | 60 (71) | K-*ras* mutation | 32/39 | 9/9 | 3/8 | 2/4 |
| | | CA 19.9 >250 U/ml | 29/39 | 5/9 | 5/8 | 2/4 |
| Other malignancies | 6 (7) | K-*ras* mutation | 0/5 | 0 | 0/1 | 0 |
| | | CA 19.9 >250 U/ml | 1/5 | 0 | 0/1 | 0 |
| Mucinous tumour | 2 (2) | K-*ras* mutation | 0 | 0 | 0 | 0/2 |
| | | CA 19.9 >250 U/ml | 0 | 0 | 0 | 0/2 |
| Endocrine tumour | 4 (5) | K-*ras* mutation | 0 | 0 | 0 | 0/4[a] |
| | | CA 19.9 >250 U/ml | 0 | 0 | 0 | 0/4[a] |
| Benign diseases | | | | | | |
| Chronic pancreatitis | 10 (12) | K-*ras* mutation | 0 | 0 | 0/3 | 0/7 |
| | | CA 19.9 >250 U/ml | 0 | 0 | 0/3 | 1/7 |
| Other | 2 (2) | K-*ras* mutation | 0 | 0 | 0 | 0/2 |
| | | CA 19.9 >250 U/ml | 0 | 0 | 0 | 0/2 |

RFLP/PCR, restriction fragment length polymorphism/polymerase chain reaction.
[a] Endocrine cells.

indicated. Final diagnoses were 15 pancreatic adenocarcinomas, 1 gallbladder carcinoma, 51 chronic pancreatitis, 3 acute pancreatitis and 1 abdominal pain. Finally, in one asymptomatic patient showing strong family history of pancreatic cancer, ERCP was performed. One of the adenocarcinoma patients had a 5-year history of alcoholic chronic pancreatitis and developed pancreatic cancer during the follow-up period (1 month after ERCP). All the procedures were in accordance with the standards of our institution's ethical committee.

### 2.2. Cytological examination

The cellular material obtained in FNA samples or pancreatic juices was examined after Papanicolaou staining and the presence of malignant cells, suspicious cells, normal appearing duct cells or insufficient material was reported.

### 2.3. Detection of K-ras codon 12 mutations

DNA was extracted following standard procedures. Mutations in codon 12 of the K-*ras* gene were detected by means of the artificial RFLP/PCR method for the restriction enzyme *Bst*NI (New England Biolabs Inc., Beverly, MA, USA). This method was used as previously described [21]. Briefly, the first-round amplification was performed using the primer DD5P: 5′TCATGAAAATGGTCAGAGAA 3′. In order to create the restriction site for the enzyme *Bst*NI [CCTGG], that is lost when a K-*ras* codon 12 mutation exists, we used the mutant primer K-*ras* 5′: 5′ACTGAATATAAACTTGTGGTAGTTGGACCT 3′ [23] for 10 cycles (44°C, 15 s; 72°C, 15 s; 92°C, 15 s). One μl of the amplified product was reamplified using a heminested reaction with mutant primers K-*ras* 5′ and K-*ras* 3′: 5′ TCAAAGAATGGTCCTGGACC 3′ [21] for 35 cycles (54°C, 15 s; 72°C, 15 s; 92°C, 15 s). The latter primer artificially introduces an internal control to assure the completion of enzymatic digestion. After polyacrylamide gel electrophoresis (PAGE) (6%) and ethidium bromide staining (0.5 mg/ml), the 143 bp band depicts the mutant allele and the 114 bp band the normal allele. The positive control was NP9, a human pancreatic carcinoma cell line, homozygous for an aspartic acid substitution at codon 12 of the K-*ras* gene. The negative control was NP18, a human pancreatic carcinoma cell line that is negative for the mutation. Positive (M) and negative (N) controls for the mutation, molecular weight markers ($\phi \times 174$, *Hae*III-digested) and controls for carryover DNA contamination were included in every experiment (Fig. 1). All samples were analysed in duplicate. This standard

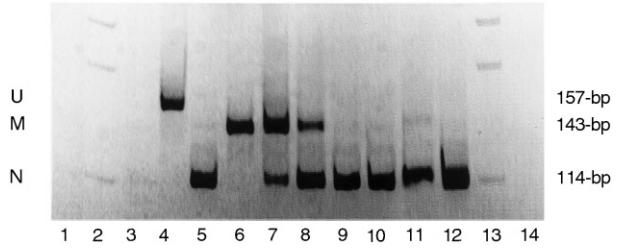


Fig. 1. Detection of K-*ras* mutation by restriction fragment length polymorphism/polymerase chain reaction (RFLP/PCR) methods. Lanes 2, 13, molecular weight markers; lane 4, uncut PCR-product (U); lanes 5, 12, negative control (N); lane 6; positive control (M); lanes 7–8, positive samples; lanes 9–11, negative samples; lanes 1, 3, 14, control samples without DNA.

RFLP/PCR method allows the detection of 1 mutant allele among 100 wild-type alleles [21].

### 2.4. CA 19.9 determination

The serum CA 19.9 concentration was measured using a commercially available two-site immunoluminometric assay (LIA-mat® CA 19.9, Byk-Sangtec Diagnostica, D-6057 Dietzenbach, Germany). The CA 19.9 present in the sample sequentially binds a solid phase antibody (coated tubes) and a monoclonal antibody conjugated with isoluminol. The light produced by oxidation of isoluminol is measured in a luminometer (425 nm). The reference value of $\leqslant 37$ U/ml was set by the manufacturer based on healthy controls.

In a previous study [20], we established the cut-off values that gave us a CA 19.9 specificity of 90%, in both clinical situations, using ROC curves. The cut-off value obtained when a pancreatic mass is present was 250 U/ml (group 1) and 100 U/ml in the absence of pancreatic mass (group 2). In this study, we prospectively validated the performance of these cut-off values when CA 19.9 is used alone or in combination with cytological examination and K-*ras* mutational analysis.

### 2.5. Statistical analysis

Statistical analysis was performed with STATISTICA for Windows (Stat Soft, Inc. Tulsa, OK, USA). Differences between the CA 19.9 means of groups defined according to the presence of K-*ras* mutations, were assessed by analysis of variance (ANOVA). Differences between two proportions were tested with a one-sided *t*-test. A $P$ value $<0.05$ was considered as statistically significant.

## 3. Results

### 3.1. Group 1: patients presenting with a pancreatic mass

Cytology offered a conclusive diagnosis in 63 of 84 (75%) cases. The cytological report was not conclusive in 21 cases (25%) (9 cases with suspicious cells and 12 cases with insufficient material Table 1. The presence of malignant cells in the FNA samples from patients with a pancreatic mass was reported in 39 of 60 pancreatic carcinomas (65%). No false-positives were detected in the remaining 24 conclusive FNAs.

Molecular analysis using the RFLP/PCR method was always feasible. K-*ras* mutations were detected in 46 of 60 FNA samples (77%) from adenocarcinomas patients and no mutations were detected in the remaining 24 FNA samples. The combined molecular and cytological approach was always informative and offered an 88% sensitivity with a 100% specificity (Table 2).

Using the cut-off value of 250 U/ml established in our previous study [20] CA 19.9 measurement identified 41 of the 60 pancreatic carcinomas (range = 314–135 200 U/ml). Additionally, high concentrations were also detected in 1 cholangiocarcinoma (1890 U/ml) and in 1 of 10 chronic pancreatitis patients presenting with a mass (337 U/ml). CA 19.9 positivity in the chronic pancreatitis patient was related to a common bile duct obstruction. The combined CA 19.9 and cytological assay was always informative and showed a sensitivity of 85% with a specificity of 92%.

#### 3.1.1. Combination of cytological analysis, K-ras mutation and CA 19.9

Since all positive cytologies carried a final diagnosis of carcinoma, the main diagnostic contribution of K-*ras* and CA 19.9 in the pancreatic carcinoma group was when the cytology was negative or not conclusive. Either test contributed to the final diagnosis of pancreatic adenocarcinoma in a total of 18 out of 60 (30%) cases: in 8 cases both K-*ras* and CA 19.9 were positive; in 6 cases K-*ras* mutations were exclusively detected and in 4 cases only high CA 19.9 values were observed. As expected, no patient with chronic pancreatitis presenting a pancreatic mass was positive for both markers. The sensitivity of the combined approach was 95% with a specificity of 92% (Table 2). Both sensitivity and efficiency of the combined approach (cytology, CA 19.9 and K-*ras*) was significantly higher than that of the tests considered alone ($P<0.005$ (for CA 19.9) and $P<0.05$

Table 2
Assay effectiveness in patients with a pancreatic mass ($n=84$)

| Assay | Sensitivity TP/(TP + FN) | Specificity TN/(FP + TN) | Predictive value of a positive test TP/(TP + FP) | Efficiency (TP + TN)/Total |
|---|---|---|---|---|
| Cytology alone | 39/(39 + 13) = 0.75 | 20/(0 + 20) = 1.00 | 39/(39 + 0) = 1.00 | (39 + 20)/84 = 0.70 |
| CA 19.9 > 250 U/ml alone | 41/(41 + 19) = 0.68 | 22/(2 + 22) = 0.92 | 41/(41 + 2) = 0.95 | (41 + 22)/84 = 0.75 |
| K-*ras* mutation alone | 46/(46 + 14) = 0.77 | 24/(0 + 24) = 1.00 | 46/(46 + 0) = 1.00 | (46 + 24)/84 = 0.83 |
| Either cytology + CA 19.9 (> 250 U/ml) | 51/(51 + 9) = 0.85 | 22/(2 + 22) = 0.92 | 51/(51 + 2) = 0.96 | (51 + 22)/84 = 0.87 |
| Either cytology + K-*ras* mutation | 53/(53 + 7) = 0.88 | 24/(0 + 24) = 1.00 | 53/(53 + 0) = 1.00 | (53 + 24)/84 = 0.92 |
| Either cytology + CA 19.9 (> 250 U/ml) + K-*ras* mutation | 57/(57 + 3) = 0.95 | 22/(2 + 22) = 0.92 | 57/(57 + 2) = 0.97 | (57 + 22)/84 = 0.94 |

TP, true-positive; FN, false-negative; FP, false-positive; TN, true-negative.

Table 3
Incidence of *K-ras* mutations by restriction fragmant length polymorphism/polymerase chain reaction (RFLP/PCR) methods and CA 19.9 determination according to cytological diagnosis in patients without a pancreatic mass ($n=72$)

| Final diagnosis | Patients ($n$) (%) | | Malignant cells | Suspicious cells | Insufficient material | Non-malignant cells |
|---|---|---|---|---|---|---|
| Malignant diseases | | | | | | |
| Pancreatic ductal adenocarcinoma | 15 (21) | K-*ras* mutation | 2/2 | 2/3 | 1/3 | 3/7 |
| | | CA 19.9 >100 U/ml | 2/2 | 3/3 | 2/3 | 3/7 |
| Other malignancies | 1 (1) | K-*ras* mutation | 0 | 0 | 0/1 | 0 |
| | | CA 19.9 >100 U/ml | 0 | 0 | 1/1 | 0 |
| Benign diseases | | | | | | |
| Chronic pancreatitis | 51 (71) | K-*ras* mutation | 0 | 0/1 | 1/10 | 4/40 |
| | | CA 19.9 >100 U/ml | 0 | 0/1 | 0/10 | 6/40 |
| Acute pancreatitis | 3 (4) | K-*ras* mutation | 0 | 0 | 0/2 | 0/1 |
| | | CA 19.9 >100 U/ml | 0 | 0 | 0/2 | 0/1 |
| Other | 2 (3) | K-*ras* mutation | 0 | 0 | 0 | 0/1 |
| | | CA 19.9 >100 U/ml | 0 | 0 | 0 | 0/2 |

(for CA19.9), respectively). No statistical differences were observed between the combinations of the two techniques. Finally, the sensitivity of the combined approach was significantly better than that of cytology and CA 19.9 ($P<0.05$), whereas no differences were observed regarding efficiency. It is of note that no differences between CA 19.9 means can be observed, using the two-way ANOVA test, when samples are grouped according to both K-*ras* mutational status and the diagnosis of pancreatic cancer and chronic pancreatitis ($P=0.267$ and 0.776 respectively).

### 3.2. Group 2: patients presenting without a pancreatic mass

In the absence of a pancreatic mass, cytology offered a low diagnostic yield and malignant cells were detected in only in 2 of 15 (13%) pancreatic carcinomas with no false positives in the remaining 57 pancreatic juice samples. However, in 20 of the 72 (28%) juices the cytological report was not conclusive: in 16 cases insufficient material was present and in 4 additional cases suspicious cells were reported (Table 3). Sensitivity, specificity, predictive value of a positive test and efficiency for cytology alone as a diagnostic tool in this group of patients are shown in Table 4.

In pancreatic juices, the sensitivity of K-*ras* mutation detection was 53% (8/15). However, in sharp contrast with pancreatic masses, K-*ras* mutations were detected in the pancreatic juices of 5 patients with chronic pancreatitis (Tables 1 and 3). In this situation, molecular analyses proved to be of limited value and cytology does not add significant information (Table 4).

In the absence of a pancreatic mass, the cut-off obtained with a 90% specificity for CA 19.9 was 100 U/ml [20]. CA 19.9 identified 10 of the 15 pancreatic adenocarcinomas (range = 133–209 000 U/ml) and one gallbladder carcinoma (16 866 U/ml) (Table 3). Combined CA 19.9 and cytological assay was always informative and showed a sensitivity of 67% with a specificity of 88% (Table 4). CA 19.9 was positive in 6 of 51 chronic pancreatitis patients (concentrations between 115 and 244 U/ml) and this was related to the common bile duct obstruction in 4 cases.

#### 3.2.1. Combination of K-ras mutation, CA 19.9 and cytological analysis

In this group of patients, the presence of mutated K-*ras* and/or CA 19.9 positivity contributed to cytological diagnosis of pancreatic adenocarcinoma in a total of 11 out of 15 cases. Of these, 3 showed exclusively

Table 4
Assay effectiveness in patients without a pancreatic mass ($n=72$)

| Assay | Sensitivity TP/(TP+FN) | Specificity TN/(FP+TN) | Predictive value of a positive test TP/(TP+FP) | Efficiency (TP+TN)/Total |
|---|---|---|---|---|
| Cytology alone | 2/(2+10)=0.17 | 44/(0+44)=1.00 | 2/(2+0)=1.00 | (2+48)/72=0.64 |
| CA 19.9 >100 U/ml alone | 10/(10+5)=0.67 | 50/(7+50)=0.88 | 10/(10+7)=0.59 | (10+50)/72=0.83 |
| K-*ras* mutation alone | 8/(8+7)=0.53 | 52/(5+52)=0.91 | 8/(8+5)=0.62 | (8+52)/72=0.83 |
| Either cytology+CA 19.9 (>100 U/ml) | 10/(10+5)=0.67 | 50/(7+50)=0.88 | 10/(10+7)=0.59 | (10+50)/72=0.83 |
| Either cytology+K-*ras* mutation | 8/(8+7)=0.53 | 52/(5+52)=0.91 | 8/(8+5)=0.62 | (8+52)/72=0.83 |
| Either cytology+CA 19.9 (>100 U/ml) +K-*ras* mutation | 13/(13+2)=0.87 | 45/(12+45)=0.79 | 13/(13+12)=0.52 | (13+45)/72=0.81 |

TP, true-positive; FN, false-negative; FP, false-positive; TN, true-negative.

K-*ras* mutations, 5 had elevation of CA 19.9 only and in 3 cases both K-*ras* and CA 19.9 were positive. Interestingly, no patients with chronic pancreatitis were positive for both markers. It is of note that the 5-year chronic pancreatitis patient that developed a pancreatic cancer 1 month after ERCP had a K-*ras* mutation in their pancreatic juice sample and a serum CA 19.9 concentration higher than 100 U/ml. The combined assays offered a sensitivity of 87% with a specificity of 79% and an efficiency of 81% (Table 4). No statistical differences were observed between combinations of the two techniques. In the absence of a mass, neither sensitivity nor efficiency of the combined approach was superior to that of cytology and CA 19.9 ($P$ = ns).

## 4. Discussion

Several studies have suggested that the detection of K-*ras* mutations can be useful for the diagnosis of pancreatic cancer. However, the addition of this molecular technique to the diagnostic work-up of these patients needs evaluation in combination with other already tested diagnostic tools. In the present study, we have studied the relative and combined contribution of the detection of K-*ras* mutations and CA 19.9 concentrations to the cytological diagnosis of pancreatic cancer when there is a clinical suspicion of pancreatic cancer corroborated by imaging diagnostic techniques. In order to more precisely define its role, we have independently analysed the two most common clinical situations in which a pancreatic cancer is suspected: when a pancreatic mass is present or when other symptoms suggest its diagnosis in the absence of a mass.

In the presence of a pancreatic mass, both K-*ras* mutation and CA19.9 concentrations contributed to pancreatic cancer diagnosis. The reported incidence of K-*ras* codon 12 mutations in pancreatic adenocarcinomas oscillates between 65 and 100% [20,24,25]. In FNA samples, mutations were detected in 77% of carcinomas. Interestingly, no false-positives were detected even in the subset of chronic pancreatitis patients with a mass. This result is in concordance with previously reported results from our group [21]. Using a cut-off value of 250 U/ml, high CA 19.9 concentrations are strongly suggestive of pancreatic cancer. Although CA 19.9 is superior to other single markers evaluated, it is not suitable for the detection of localised disease since high concentrations usually occur in patients with advanced cancer [17,26,27].

The combination of the three assays offered the best sensitivity; in 18 cases out of 60 a positive K-*ras* or CA 19.9 result contributed to the cytological diagnosis. While K-*ras* mutations in FNAs are specific for pancreatic cancer, high CA19.9 values are not (one cholangiocarcinoma and one chronic pancreatitis showed CA 19.9 concentrations $>250$ U/ml), limiting its diagnostic contribution. Previously, only one study [25] analysed the usefulness of combining K-*ras* mutational analysis and CEA determinations in FNAs, suggesting that a combined approach could be most useful and avoid the need of a second procedure to confirm diagnosis.

In pancreatic juices in the absence of a pancreatic mass, the situation is somewhat different. K-*ras* mutations were present in 53% of patients with pancreatic carcinoma, but false-positives were detected in 10% of patients with chronic pancreatitis. These results are in concordance with data from our previous study [22] and from other groups in which the detection of K-*ras* mutation oscillates between 0 and 37% [8–11]. Therefore, the clinical usefulness of K-*ras* mutations in the early diagnosis of pancreatic cancer still remains unknown. In some cases, they will never develop pancreatic cancer [8,28], while in others it has been the earlier diagnostic indication of cancer [10,29]. In agreement with the latter observations, one of the chronic pancreatitis patients harbouring a mutation in his pancreatic juice finally developed a pancreatic cancer. In the absence of a mass, CA 19.9 concentrations higher than 100 U/ml increased the sensitivity of cytology analysis up to 67%. When combining the three assays, the sensitivity rose to 87%. A positive K-*ras* and/or CA 19.9 contributed to cytological diagnosis of pancreatic cancer in 11 cases out of 15; in 3 cases both markers were positive. Nevertheless, in spite of the limited clinical utility of CA 19.9 due to the presence of false-positives, CA 19.9 is apparently more useful than the detection of K-*ras* mutations in contributing to cytological diagnosis of pancreatic juices. It is of note that these false-positives would have disappeared if a 250 U/ml cut-off value was chosen. However, this would affect the true-positives in this group (2 pancreatic cancer patients with CA 19.9 concentrations of 133 and 234 U/ml would have not been detected), lowering the sensitivity from 67% (10/15) to 53% (8/15).

Interestingly, no patients with chronic pancreatitis were positive for both markers, suggesting that K-*ras* mutations and low CA 19.9 values are indicative of the absence of a pancreatic carcinoma. In sharp contrast, when no chronic pancreatitis is evident, the same combination is strongly suggestive for pancreatic cancer. Both observations suggest that the combination of the three markers (cytology, K-*ras* and CA 19.9) may be useful in distinguishing between diagnoses of pancreatic cancer and chronic pancreatitis.

In conclusion, K-*ras* mutational analysis and CA 19.9 determination enhanced the diagnostic sensitivity of cytological evaluation in patients with clinical suspicion of pancreatic cancer. When a pancreatic mass was present, cytology offered a high sensitivity, but with a significant number of inconclusive results. In this context, K-*ras* mutational analysis offered a highly specific

test. In the absence of a pancreatic mass, CA 19.9 (cut-off 100 U/ml) increased the sensitivity of cytology while K-*ras* mutations contributed little to the clinical decision-making process. Moreover, both tests contributed to the clinical decision process when the cytological report was not conclusive due to the presence of suspicious cells, when cellular material was insufficient or the presence of normal-appearing duct cells was reported. However, the relative contribution of K-*ras* and CA 19.9 varied depending upon the presence or absence of a pancreatic mass. Further studies in other series of patients are needed to validate these observations.

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