Detection of c-Ki-ras gene codon 12 mutations from pancreatic duct brushings in the diagnosis of pancreatic tumours

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Abstract

Differential diagnosis of pancreatic cancer and chronic pancreatitis is sometimes difficult and cytological examination of brushings or aspirated material collected during endoscopic retrograde cholangiopancreatography (ERCP) remains disappointing. As point mutations in codon 12 of the c-Ki-ras 2 gene are found in most pancreatic adenocarcinoma and not in chronic pancreatitis, this study analysed prospectively the presence of these mutations in brushing samples collected during ERCP in 45 patients (26 males, 19 females) showing a dominant stricture of the main pancreatic duct at pancreatography: 24 with pancreatic adenocarcinoma, 16 with chronic pancreatitis, and five intraductal mucin hypersecreting neoplasms. Twenty of 45 patients presented equivocal ERCP findings that did not permit a definite diagnosis. Ki-ras mutations at codon 12 were detected using a rapid and sensitive method based on polymerase chain reaction mediated restriction fragment length polymorphism analysis and confirmed by direct sequencing of polymerase chain reaction products. Results were compared with those provided by routine brush cytology. A definitive diagnosis was established for each patient. Mutations were detected in 20 of 24 patients with pancreatic adenocarcinoma (83%), but in none of the chronic pancreatitis patients and intraductal mucin hypersecreting neoplasms, irrespective of their location. By contrast, only 13 of 24 pancreatic adenocarcinoma (54%) were detected by conventional cytological examination, which yielded four false negative and seven non-contributive results. Sensitivity, specificity, and accuracy of molecular biological and cytological methods were 83%–76%, 100–83%, and 90%–58%, respectively. Notably the mutations could be detected in six patients with small tumour size (≤2 cm). In conclusion, Ki-ras analysis performed on pancreatic brushing samples is an efficient procedure, more accurate than cytology in the diagnosis of pancreatic adenocarcinoma, and highly specific in the differentiation between neoplastic and chronic inflammatory ductal changes, especially in patients showing inconclusive ERCP findings.

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Keywords: Ki-ras gene mutation, endoscopic retrograde cholangiopancreatography, pancreatic cancer, chronic pancreatitis.

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Despite considerable advances in imaging techniques, early diagnosis of pancreatic adenocarcinoma particularly in the setting of chronic pancreatitis remains elusive. Clinicians are often faced with unresectable tumours with a rapidly fatal outcome.[1] A recent report of increased pancreatic adenocarcinoma risk in chronic pancreatitis patients has renewed the interest in early tumour diagnosis and in the differentiation between neoplastic and chronic inflammatory ductal changes.[2]

No currently available diagnostic modality is infallible. Endoscopic retrograde cholangiopancreatography (ERCP) is highly accurate in the diagnosis of pancreatic adenocarcinoma and its distinction from chronic pancreatitis, providing both sensitivity and specificity of more than 90%.[3–4] While endoscopic ultrasonography has a high yield in small tumour (≤25 mm) detection it lacks adequate specificity.[5–6] Cytological confirmation of radiographic findings is usually required, however, for the definitive diagnosis. The accuracy of cytological examinations performed on main pancreatic duct brushings or pancreatic juice sampled during ERCP is limited by a low sensitivity (ranging from 50 to 76%).[7–9]

The Ki-ras oncogene is activated by specific point mutations restricted to codon 12 in 75 to 100% of pancreatic adenocarcinoma.[10–13] Interestingly, these mutations seem to occur at an early stage of the neoplasm as well as a preneoplastic change.[14–15] In the era of molecular biology, this finding might be sought to improve our diagnostic approach. An attempt at diagnosis, based on detection of this genetic change has already been performed in fine needle tumour aspirates or pancreatic juice samples with promising preliminary results.[16 17]

In this study, we prospectively analysed the frequency of the codon 12 changes of c-Ki-ras 2 gene in brushing samples collected during ERCP in patients showing a dominant stricture of the main pancreatic duct related to pancreatic adenocarcinoma or chronic pancreatitis. As a significant number of subjects presented equivocal ERCP findings, we compared the results of genetic analysis with those obtained by routine cytological examination in an attempt to differentiate malignant from benign lesions. Similarly, we studied five

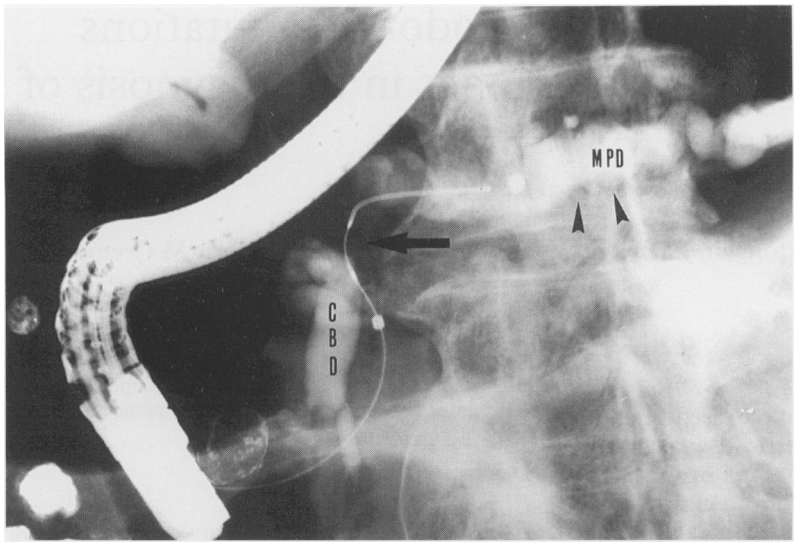

Figure 1: Cytology brush (large arrow) strategically placed within a main pancreatic duct stricture. The main pancreatic duct is dilated in its body segment above the stricture (arrowheads) next to the flexible guide tip. CBD=common bile duct.

additional cases of intraductal mucin hypersecreting neoplasms, exhibiting ductal abnormalities at pancreatography. Because these tumours are considered to have a good prognosis but may undergo malignant transformation,[18] we found it of interest to investigate a new genetic marker, which might predict their behaviour and prognosis.

Methods

PATIENTS

From November 1992 to December 1993, 45 patients (26 males, 19 females) receiving ERCP at our hospital were included: 24 patients with pancreatic adenocarcinoma (mean age: 69·1 years, range 41–79), 16 with chronic pancreatitis (mean age: 49·4 years, range 35–73), and five with intraductal mucin hypersecreting neoplasm (mean age: 62·5 years, range 55–70). All exhibited a dominant stricture of the main pancreatic duct or duct clogging with mucoid material (in the case of intraductal mucin hypersecreting neoplasm). Twenty of 45 patients presented equivocal ERCP findings that did not permit a definite diagnosis of malignancy (see Tables I–III).

For each patient, a definitive diagnosis was established. The diagnosis of pancreatic adenocarcinoma was confirmed histologically in 16 of 24 patients, including 14 ductal adenocarcinoma and two cystadenocarcinoma. Eight other patients had unresectable pancreatic carcinoma as evidenced by endoscopic ultrasonography or computed tomography and by a rapidly worsening clinical course (detailed in Table II).

The diagnosis of chronic pancreatitis was supported by standard techniques (biochemical, computed tomography, and endoscopic ultrasonography and mainly by a favourable clinical outcome (detailed in Table I). All patients were clinically and radiologically followed up for at least six months (mean (SD) follow up: 9·3 (2·2) months) without evidence of a worsening status.

Diagnosis of intraductal mucin hypersecreting neoplasm was established histologically after laparotomy in two of five patients; three patients had typical previously described clinical, endoscopic, and cytological findings[18] – that is, relapsing pancreatitis like symptoms, widely open ampulla of Vater filled with mucoid material, multicystic dilations of the main pancreatic duct with patchy filling defects at pancreatography, and presence of exfoliated mucin rich tall columnar cells in abundant mucoid material at cytological examination.

SAMPLING TECHNIQUE

Diagnostic ERCPs were performed using an Olympus TJF-100 duodenoscope; lesions were identified after injection of iodinated contrast agent. Pancreatic duct brushings were collected with a Geenen cytology brush system (Wilson-Cook, Winston-Salem NC), fluoroscopically positioned through the suspected lesion (Fig 1). The brush was moved briskly several times over epithelium and then withdrawn into the outer sheath, aspirating additional epithelial cells. The procedure avoided contamination of the sample with cells from irrelevant sites (for example, duodenal tract) during brush withdrawal. Brushings were systematically obtained in duplicate for genetic analysis and routine cytological examination. Specimens were immediately collected in 5 ml of phosphate buffered saline (PBS) at 4°C for DNA analysis or smeared on slides, fixed in 85% isopropyl alcohol, and stained by the Papanicolaou technique for concomitant standard cytological examination. Adequate material was obtained in each patient.

DNA PREPARATION

Samples were centrifuged at 2000 rpm for 15–20 minutes to collect cells. Cell pellets were resuspended in 50–100 µl of a TRIS 0·1 M, EDTA 0·05 M, pH 8 buffer containing 0·1 mg/ml proteinase K (Sigma) and incubated at 42°C for two hours. Proteinase K was then inactivated by boiling the samples at 95°C for 10 minutes. Proteinase K digests were centrifuged at 10 000 rpm for five minutes and 10 µl of the supernatant was submitted to a polymerase chain reaction amplification.

POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

c-Ki-ras mutations at codon 12 were detected using restriction fragment length polymorphism artificially introduced by the use of modified polymerase chain reaction primers as previously described[19 20]; the two primers used were a 22 mer (5′ TAAACTTGTGGTAGT TGGAGCC 3′) and a 20 mer (5′ TCTATT GTTGGATCATATTC 3′); substitution of C for T at the 3′ end of the 22 mer introduces an artificial Msp I restriction site (CCGG) when normal Ki-ras sequences are amplified but not when there is a mutation in the first or second position of codon 12. Thus, genomic DNA was amplified by the polymerase chain

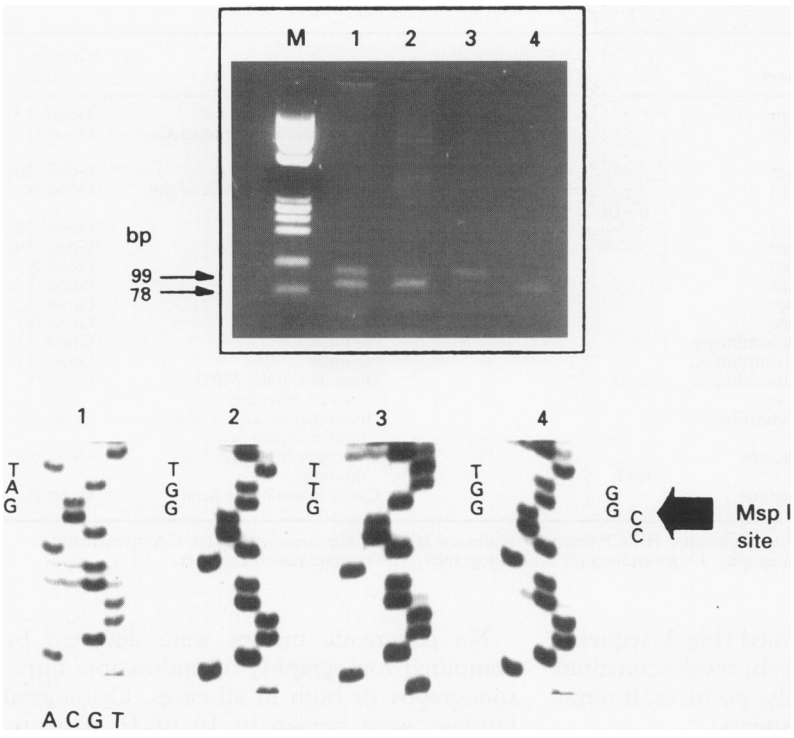

Figure 2: Detection of point mutations at codon 12 by polymerase chain reaction-restriction fragment length polymorphism analysis and direct sequencing (DS): lane 1: *both 99 bp and 78 bp bands of similar intensity=positive result confirmed by DS: GGT→GAT;* lane 2: *faint 99 bp band/dominant 78 bp band=false positive result invalidated by DS: GGT;* lane 3: *dominant 99 bp band/very faint 78 bp band=positive result confirmed by DS: GGT→GTT;* lane 4: *single 78 bp band=negative result verified by DS: GGT. M=DNA size ladder; CCGG=Msp I cutting site in normal Ki-ras sequence.*

reaction technique using the two primers in a 100 µl amplification reaction containing 10 mM TRIS-HCl (pH 8·3), 50 mM KCl, 2·5 mM $MgCl_2$, 100 µM of each dATP, dCTP, dGTP, dTTP, and five units of Ampli TAQ DNA polymerase. Reactions were incubated in a Perkin-Elmer/Cetus DNA thermal cycler for 35–40 cycles (denaturation: 1 min at 94°C; annealing: 1 min at 55°C; extension: 1 min 30 at 72°C) generating DNA fragments of 99 base pairs (bp). Ten µl of the amplified DNA were then digested with 20 and 40 units Msp I, at 37°C for two and 16 hours respectively. Identification of digested polymerase chain reaction products was obtained by separation on 4% agarose MP (Boehringer-Mannheim) gel, staining with ethidium bromide, and visualisation under ultraviolet light. In the presence of the normal Ki-ras gene, the Msp I digestion generated a 78 bp and a 21 bp fragment; by contrast when the Msp I cutting site was changed by a point mutation at codon 12, a persistent 99 bp fragment was seen. Amplified samples from normal colonic mucosa and from the colonic carcinoma SW 480 cell line (which is known to carry the mutation at codon 12) were treated simultaneously as normal and positive controls, respectively. This method was reported to have a great sensitivity, detecting the presence of a point mutation in 5–10% of the cell population, although the nature of the mutation was not identified.[19 20] Results can be obtained within 24 hours.

As this rapid method can in certain cases yield ambiguous results, caused by partial digestion (that is, when a faint 99 bp band is seen after Msp I digestion), we performed direct sequencing to validate it; all polymerase chain reaction products showing persistence of a clear or faint 99 bp band after digestion were submitted for direct sequencing to confirm the presence of point mutations at codon 12.

DIRECT SEQUENCING

In brief, polymerase chain reaction products were ligated to pCR II vector using a TA Cloning Kit (Invitrogen Corporation, San Diego, CA, USA). One shot INV α F′ competent cells (Invitrogen) were transformed with the pCR II vector containing polymerase chain reaction products using the same kit and plated on Luria-Bertani medium-Agar plates with ampicillin 50 µg/ml and X-Gal 40 mg/ml for selection.

Selected colonies were grown in Luria-Bertani medium+ampicillin medium for DNA plasmid isolation using a Quiagen plasmid mini Kit (Quiagen Inc, Chatsworth, CA, USA). Polymerase chain reaction products were directly sequenced by the dideoxy chain termination sequencing method[21] using Sequenase version 2·0 DNA sequencing Kit (United States Biochemical, Cleveland, Ohio) according to the manufacturer's instructions.

CYTOLOGICAL EXAMINATION

Results provided by Ki-ras analysis were compared with those obtained by routine cytological examination. Cytological findings were classified by a pathologist blinded to the genetic analysis data, as malignant (=positive for malignant cells – malignancy deemed certain), suspicious (=presence of nuclear and cellular atypia suspicious of malignancy), benign (=negative for malignant cells) or not contributive (=insufficient cellular material collected or not adequate fixation).

Results

VALIDATION OF THE POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS METHOD

After separation of the digested polymerase chain reaction products on agarose MP gel, four types of results were seen (Fig 2): (a) both 99 and 78 bp bands of similar intensity (lane 1), n=16 patients; (b) faint 99 bp band and a dominant 78 bp band (lane 2), n=8 patients; (c) dominant 99 bp band and a faint 78 bp band (lane 3), n=4 patients; (d) one single 78 bp band (lane 4), n=17 patients.

In all 20 patients showing a clearly visualised 99 bp band (type (a) and (c) results), mutations at codon 12 were confirmed by direct sequencing (Fig 2, sequences 1 and 3); the more frequent mutation detected was GTT in place of GGT.

By contrast, in the eight patients with type (b) results, we failed to detect any mutation (Fig 2, sequence 2). In addition, direct sequencing was performed in five patients with type (d) results where normal Ki-ras codon 12

TABLE I *Diagnostic findings and clinical outcome in patients with chronic pancreatitis (n=16)*

	Location of the stricture(s) at ERCP	*Cytology*		*Ki-ras mutations at codon 12*	*CT/EUS*	*Outcome (month)*
1	Head†	Benign		–	Normal	Good (14)
2	Head and body†	Benign		–	Cystic lesion of the head-Ca^{++} (about 25 mm)	Good (11)
3	Head-body*	Benign		–	No mass-Ca^{++}	Good (10)
4	Head*	Benign		–	Heterogeneous aspect of the head. No mass	Good (11)
5	Head and body*	Benign	n=10	–	No mass-Ca^{++}	Good (10)
6	Head*	Benign		–	No mass-Ca^{++}	Good (10)
7	Body†	Benign		–	No mass-Ca^{++}	Good (9)
8	Body*	Benign		–	No mass-Ca^{++}	Good (11)
9	Head*	Benign		–	Normal	Good (6)
10	Head and body†	Benign		–	No mass-Ca^{++}	Good (6)
11	Head*	Not contributive		–	No mass-Ca^{++}	Good (11)
12	Head†	Not contributive		–	No mass	Good (11)
13	Head*	Not contributive	n=4	–	Dilatation of the MPD and its side branches	Good (9)
14	Body-tail†	Non contribute		–	Heterogenous aspect of the body-atrophic tail	Good (9)
15	Head and body†	Suspicious	n=2	–	Heterogeneous aspect of the body	Good (6)
16	Head†	Suspicious		–	Cystic lesion of the head	Good (6)

*ERCP findings consistent with benign changes; †ERCP findings=equivocal aspect; malignancy suspected; CA^{++}pancreatic calcifications. CT=computed tomography, EUS=endoscopic ultrasonography, MPD=main pancreatic duct.

sequence was well confirmed (Fig 2, sequence 4). Considering that type (b) results constitute the potential source of false positives, it represents 17% (eight of 45 patients).

Results of Ki-ras analysis reported below are provided using both methods.

KI-RAS MUTATIONS AT CODON 12 IN PANCREATIC BRUSHINGS

Chronic pancreatitis

Table I details the diagnostic findings and clinical outcomes of chronic pancreatitis patients (n=16). In eight of 16 patients with chronic pancreatitis, the diagnosis of benign stricture(s) was clearly suggested by ERCP while eight patients presented equivocal ERCP findings.

No pancreatic masses were detected by computed tomography, or endoscopic ultrasonography or both in all cases. Cytological findings were benign in 10 of 16 patients, non-contributory in four, and suspicious in two.

A definitive diagnosis of chronic pancreatitis was established by a regular follow up; all patients had a benign course (mean (SD) follow up: 9·3 (2·2) months, range 6–14) and none showed evidence of Ki-ras mutations at codon 12.

Pancreatic adenocarcinoma

Table II shows the data on pancreatic adenocarcinoma patients (n=24): 14 of 24 patients showed ERCP findings strongly suggestive of malignancy while 10 had inconclusive pancreatography.

TABLE II *Comparison of point mutations detection with the cytological and radiological findings in patients with pancreatic adenocarcinoma (n=24)*

Patients	*Location of the stricture at the ERCP*	*Cytology*		*Ki-ras mutated at codon 12*	*Tumour size‡*	*Diagnostic confirmation*
1	Head†	Malignant		+	≤2 cm	Ductal PAC
2	Head*	Malignant		+	ND	Ductal PAC
3	Head*	Malignant		+	≤2 cm	Ductal PAC
4	Head*	Malignant		+	>2 cm	Ductal PAC
5	Head*	Malignant		+	>2 cm	Clinical course
6	Head-body†	Malignant	n=12	+	>2 cm	Cystadenocarcinoma
7	Head*	Malignant		+	ND	Ductal PAC
8	Head*	Malignant		+	>2 cm	Ductal PAC
9	Head-body†	Malignant		+	>2 cm	Clinical course
10	Head†	Malignant		+	>2 cm	Ductal PAC
11	Head*	Malignant		+	>2 cm	Cystadenocarcinoma
12	Head*	Malignant		–	>2 cm	Ductal PAC
13	Body†	Suspicious	n=1	+	≤2 cm	Ductal PAC
14	Head*	Benign		+	>2 cm	Clinical course-liver and bone metastases
15	Head*	Benign	n=4	+	>2 cm	Clinical course
16	Head†	Benign		+	≤2 cm	Ductal PAC
17	Body†	Benign		–	Non contributive acute pancreatitis	Ductal PAC
18	Body-tail†	Not contributive		+	≤2 cm	Ductal PAC
19	Head*	Not contributive		+	>2 cm	Clinical course
20	Body†	Not contributive		+	>2 cm	Clinical course neoplastic ascites
21	Body†	Not contributive	n=7	+	≤2 cm	Ductal PAC
22	Head*	Not contributive		+	>2 cm	Ductal PAC
23	Body-tail*	Not contributive		–	>2 cm	Clinical course
24	Head*	Not contributive		–	>2 cm	Clinical course liver metastases

*ERCP findings, malignant stricture strongly suspected; †ERCP findings, equivocal aspect, malignancy suggested; ‡assessed by EUS or computed tomography.
ND=not done because of clinical evidence of advanced tumour stage.
PAC=pancreatic adenocarcinoma.

TABLE III *Diagnostic findings and clinical outcome in patients with intraductal mucin hypersecreting neoplasm (n=5)*

	Location of the lesions at ERCP*	*Cytology*	*Ki-ras mutations at codon 12*	*CT/EUS*	*Outcome (month)*
1	Tail§	Benign, mild dysplasia†	–	Dilated MPD-heterogeneous mass of the tail	Good (12) after caudal pancreatectomy
2	Head§	Benign, mild dysplasia†	–	Multicystic dilatations of MPD	Good (14) after duodenopancreatectomy
3	Body‡	Benign, no atypia	–	Enlarged MPD	Good (11)
4	Head‡	Benign, no atypia	–	Cystic dilatations of the secondary duct	Good (9)
5	Head‡	Benign, no atypia	–	Parenchymatous atrophy-Ca	Good (11)

*=Substenosis+dilatations of the MPD and its side branches, mucin plugs; †=confirmed by histological examination; ‡=ERCP findings consistent with benign changes; §=ERCP findings, equivocal aspect; malignancy suspected. Other abbreviations as in Table I.

Brushing samples were obtained from all strictures and DNA analysis showed the presence of the mutations in 20 of 24 patients (83%) with pancreatic adenocarcinoma, including two of two cystadenocarcinoma. By contrast, malignancy was cytologically diagnosed or suspected in only 13 of 24 patients (54%). Four of 24 patients (17%) had benign changes (false negative cytology) and seven of 24 (29%) non-contributive tests resulting from insufficient material or inadequate fixation (inconclusive cytology). When comparing both cytological and molecular methods, Ki-ras analysis confirmed malignant or suspicious cytological findings in 12 of 13 cases and failed in one. Moreover, molecular biological method detected Ki-ras mutation in eight of 24 patients with false negative (n=3) or inconclusive (n=5) cytological findings. Three patients with pancreatic adenocarcinoma had both negative genetic and cytological findings. Patient 17 with non-contributive ERCP and endoscopic ultrasonography findings required laparotomy for definitive diagnosis (Table II). Notably mutations were detected in six patients with small tumours (≤2 cm), which were successfully resected.

Intraductal mucin hypersecreting neoplasms

Five patients with intraductal mucin hypersecreting neoplasm did not have point mutations (Table III). Two of them were suspected to have malignant degeneration and therefore had surgical resection. Histological tests showed intraductal mucin hypersecreting neoplasm with mild to moderate dysplasia.

Three other older patients, with good clinical status did not experience complications such as secondary obstructive pancreatitis and are followed up by imaging techniques, including ERCP, endoscopic ultrasonography, and computed tomography, and brush cytology (mean (SD) follow up 11·4 (1·6) months, range 9–13). In the five cases, the tumour was thus considered as benign.

VALUE OF KI-RAS CODON 12 MUTATIONS DETECTION IN THE DIFFERENTIAL DIAGNOSIS BETWEEN PANCREATIC ADENOCARCINOMA AND CHRONIC PANCREATITIS

Table IV summarises the comparison of both molecular and cytological analysis in patients with pancreatic adenocarcinoma and chronic pancreatitis (n=40) in terms of sensitivity, specificity, positive and negative predictive values, and accuracy.

Accuracy of Ki-ras analysis was significantly higher.

TABLE IV *Comparison of Ki-ras analysis and brush cytology in patients with chronic pancreatitis and pancreatic adenocarcinoma (n=40)*

	*Ki-ras analysis**	*Brush cytology†*			
	Cases with mutation (n)	*Malignant*	*Suspicious*	*Benign*	*Not contributive*
Chronic pancreatitis (n=16)	0	0	2	10	4
Pancreatic adenocarcinoma (n=24)	20	12	1	4	7
Sensitivity (%)	83	76			
Specificity (%)	100	83			
Positive predictive value (PPV)(%)	100	86			
Negative predictive value (NPV)(%)	80	71			
Accuracy (%)	90‡	58			

*=including polymerase chain reaction-restriction fragment length polymorphism analysis+direct sequencing; †=for the calculation of sensitivity, specificity, PPV, NPV, and accuracy, malignant and suspicious findings='positive' results; benign findings='negative' results.
‡=$p \leq 0{\cdot}01$, using Fisher's exact test.

Discussion

ERCP is one of the best imaging techniques in the clinical diagnosis of pancreatic cancer and differentiation from chronic pancreatitis.[3 4] Differentiating pancreatic adenocarcinoma from chronic pancreatitis can be quite difficult particularly when both diseases coexist. In our study, a significant number of patients showed equivocal ERCP findings and were deliberately selected because they required further investigations for a definitive diagnosis. This explains the high number of patients with inconclusive ERCP. Therefore, we have attempted to compare conventional cytological examination and detection of Ki-ras mutations in specimens collected by endoscopic brushings.

Cytological examination of specimens obtained by endoscopic retrograde brushings or catheter aspiration has a reported sensitivity ranging from 50 to 76%, thus limiting its usefulness as a definitive diagnostic test.[7–9] Similarly, the yield of cytological diagnosis in our study remains limited with a low accuracy (58%). Several factors could explain these disappointing results: cytological distinction between chronic pancreatitis and pancreatic adenocarcinoma is occasionally difficult because chronic pancreatitis can induce morphological changes similar to those seen in well differentiated adenocarcinoma.[22] This explains the suspicious results found in two

chronic pancreatitis patients, which were not confirmed by the clinical and radiological course and invalidated by Ki-ras analysis. Accuracy of cytology examination also depends on the cytologist's expertise, quality, and number of cells, which may be damaged by contrast medium or inadequate fixation.[9]

The recent report of Ki-ras oncogene point mutations in a high percentage of pancreatic adenocarcinoma has triggered a new wave of interest in this potential genetic marker for early diagnosis of malignancy. Several molecular biological methods using the polymerase chain reaction technique have been reported,[11-13 17] capable of detecting point mutations of Ki-ras codon 12. In this study, we have chosen to use the rapid and sensitive polymerase chain reaction-restriction fragment length polymorphism analysis method. This method uncovers codon 12 point mutations, present in only 5% of the cell population studied, composed, among others, of neoplastic, normal duct, and inflammatory cells.[19 20] Polymerase chain reaction-restriction fragment length polymorphism results are available within 24 hours, offering a rapid screening test in clinical practice. One of the possible drawbacks of this technique, however, is the yield of false positive results caused by incomplete digestion of polymerase chain reaction products. In our study, the rate of false positive results was 17%, which invariably corresponded to the persistence of a faint undigested 99 bp band. As this type of result is inconclusive, it requires confirmation by another method. Therefore, direct sequencing of polymerase chain reaction products was successfully applied to validate our positive and equivocal data. This technique is more time consuming and requires about eight days to achieve.

This study shows that Ki-ras analysis, combining the rapid test and the confirmation method when necessary, is much more accurate than brush cytology. The main advantages of the technique are its objectivity and the lack of dependence on cell integrity and number. In four of 24 cases of pancreatic adenocarcinoma, however, point mutations were not shown. This may result from the absence of Ki-ras mutations in the neoplastic cells as seen in some pancreatic adenocarcinoma or in non-ductal pancreatic adenocarcinoma. Incorrect positioning of the cytology brush, restricting efficient brushing of the neoplastic site is also possible and could explain negative results obtained from both methods in three patients.

The search for Ki-ras mutations in pancreatic ductal cells seems promising in the differential diagnostic of chronic pancreatitis and pancreatic adenocarcinoma particularly when the second occurs in a setting of chronic pancreatitis. This diagnostic challenge is of utmost clinical importance, given the recently shown increased pancreatic adenocarcinoma risk in pancreatic adenocarcinoma patients[2] and the frequency of inconclusive pancreatographic and cytological findings. In an appreciable number of our patients showing equivocal ERCP findings, Ki-ras analysis discriminated between benign and malignant strictures more specifically than cytology. Interestingly, as an increasing percentage of chronic pancreatitis patients have therapeutic endoscopic examinations, DNA analysis of ductal cells collected during these procedures might help in early detection of neoplasia. Ki-ras activation is reported to occur as an early event in pancreatic duct carcinogenesis as seen in the Syrian golden hamster model and as suggested by the frequency of Ki-ras codon 12 mutations (about 50%) in mucous cell hyperplasia and in papillary hyperplasia, found in Japanese patients.[14 15] In this study, we found the mutation in six patients with small size tumours ($\leq$2 cm). It is critical to assess in the future the effectiveness of this approach in early neoplasia detection. A longterm study of Ki-ras mutations incidence in chronic pancreatitis patients and their relation to clinical, radiological, and cytological changes would be of interest.

A similar approach might be applicable to intraductal mucin hypersecreting neoplasm, although Ki-ras mutation incidence in this disease is controversial and based on a limited number of patients. Differences exist between Japanese and occidental reports. The first[23 24] found Ki-ras mutations in respectively three of five and five of eight patients with intraductal papillary neoplasms while the second[13] did not see any mutation in a series of five patients. Our results did not show the presence of the mutated Ki-ras gene in the five patients with intraductal mucin hypersecreting neoplasm, which is similar to intraductal papillary neoplasm.[18 25] In this small series, Ki-ras analysis did not permit discrimination of chronic pancreatitis from intraductal mucin hypersecreting neoplasm while cytological findings were more suggestive. It should be emphasised, however, that none of our intraductal mucin hypersecreting neoplasms had histological or cytological features of severe dysplasia or atypia and were thus considered to have a favourable prognosis. As this type of neoplasm has a low malignant potential but is often responsible for the development of secondary obstructive pancreatitis,[18 25 26] we currently advocate tumour resection in young patients and serial follow up in older ones (above 70 years) by computed tomography, ERCP, and repeated cytological examinations of pancreatic juice or brushing samples. It might thus be useful to follow up such patients similarly using Ki-ras gene analysis.

In conclusion, molecular analysis of Ki-ras gene can be successfully performed on ERCP specimens. A first screening test can be obtained within 24 hours by polymerase chain reaction-restriction fragment length polymorphism analysis but the presence of mutations should be confirmed by direct sequencing in ambiguous cases. This molecular biological approach seems more accurate than cytology for detection of neoplastic cells and may represent an interesting addition to the diagnostic procedures for pancreatic cancer, especially in patients with ERCP and cytological findings

that do not provide conclusive evidence of malignancy.

Whether this approach might be useful in detecting malignant changes earlier remains to be evaluated.

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