Molecular markers for diagnostic cytology of neoplasms in the head region of the pancreas: mutation of K-ras and overexpression of the p53 protein product

J M van Es, M M Polak, F M van den Berg, T B Ramsoekh, M E Craanen, R H Hruban, G J A Offerhaus

Abstract

Aims—To determine the potential efficiency of molecular markers specific for neoplastic change—mutations of the K-ras oncogene and the p53 tumour suppressor gene—in diagnosing pancreatic carcinoma.

Methods—Archival cytology samples obtained from 17 patients with established pancreatic carcinoma were assayed for alterations in K-ras and p53. To detect changes in p53 expression, immunocytochemistry with polyclonal antibody CM1 was performed on the archival cytology slides after destaining. Mutations in K-ras codon 12 were then analysed on the scrapings of the same slides using mutant enriched polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism analysis with allele specific oligonucleotide hybridisation for confirmation and characterisation.

Results—False negative results were recorded for five of the cytology slides when compared with p53 immunostaining of the surgical resection specimen. These five cases had been stained previously with Giemsa which interacts adversely with the immunostaining in contrast to the Papanicolaou procedure. The K-ras codon 12 mutations followed the well established distribution frequency and spectrum for pancreatic cancer and corresponded with the findings in the resection specimens in all cases. Two scrapings yielded insufficient DNA for PCR. Importantly, for two cases with an inconclusive cytology diagnosis on routine light microscopy, the diagnosis was confirmed by one of the molecular markers. The application of the molecular markers increased the diagnostic accuracy of cytology in this small study from 76 to 89%.

Conclusions—The study indicates that assessment of alterations in the K-ras and p53 genes may be a valuable adjunct to diagnostic cytopathology of the head region of the pancreas, although there are

some difficulties which will have to be overcome.

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Pancreatic cancer is a major contributor to cancer related death in the West.¹ Most patients with pancreatic cancer present with obstructive jaundice, abdominal discomfort and weight loss,¹ and are usually evaluated by endoscopic retrograde cholangiopancreatography (ERCP) and computed tomography (CT). Cytology specimens can be collected during the above procedures, either by brush cytology or fine needle aspiration.¹² Although these cytology specimens can often be used to establish a definitive diagnosis of cancer, it may be difficult to distinguish between reactive and neoplastic changes. In about 30% of cases it is impossible to reach a definitive diagnosis.¹

Molecular markers specific for neoplastic disease could potentially improve the diagnostic yield of cytology and thus avoid unnecessary delay in treating patients. Mutations in the K-ras oncogene and the p53 tumour suppressor gene are two such potential markers for pancreatic cancer.^{3,4} K-ras oncogene mutations occur in 80 to 90% of pancreatic carcinomas and are equally prevalent in cancers of the head, body and tail of the pancreas.^{5,6} These mutations may occur relatively early in the development of pancreatic cancer,⁷ detection of which could improve prognosis. K-ras gene mutations are usually limited to one codon and therefore can be detected using a small number of probes.⁶ Recently developed enrichment techniques can be used to detect rare mutant cells in small samples such as cytology specimens.^{6,7}

In contrast to K-ras mutations, p53 tumour suppressor gene mutations occur at multiple sites, mostly in exons 5 to 8,⁸ making mutational analysis of p53 more difficult. A feature that facilitates the study and potential use of p53 as a molecular marker is the stability of the mutant p53 protein product, which can be detected immunohistochemically.⁹⁻¹¹ p53 gene

Department of Pathology, Academic Medical Centre, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
J M van Es
M M Polak
F M van den Berg
T B Ramsoekh
G J A Offerhaus

Division of Gastroenterology
M E Craanen

Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
R H Hruban

Correspondence to:
Dr G J A Offerhaus.

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Figure 1 Immunocytochemistry of representative cell specimens following PAP staining. A: Human colon cancer cell line SW480; B: cytological specimen from case 8. p53 overexpression can be seen as brown nuclear staining.

mutations are present in 50 to 70% of pancreatic carcinomas, and almost exclusively in invasive tumours. In contrast to *K-ras* mutations, mutant p53 is seldom found in in situ lesions.¹² This suggests that changes in the p53 gene occur late in carcinogenesis and therefore p53 is a relatively specific marker for malignancy.

Methods

Surgical and cytology specimens from 17 patients (nine men and eight women; mean age 60 years, range 40–80 years) with adenocarcinoma of the pancreas were retrospectively analysed. Inclusion criteria comprised a definitive diagnosis based on examination of a surgical resection specimen, at least two qualitatively good cytology glass slides collected prior to the final diagnosis (one for this study

and the other to be archived), and a diagnosis of adenocarcinoma of the head region of the pancreas. Seven of the cytology specimens had been air-dried, fixed in methanol and stained with Giemsa. The remaining 10 specimens had been fixed in 100% ethanol and stained according to the Papanicolaou (PAP) procedure. All histological material had been routinely fixed in 4% phosphate buffered formalin and embedded in paraffin wax. Sections (5 µm) were mounted on organosilane coated glass slides and dried overnight at 56°C. The anti-p53 polyclonal antibody CM1 was used for immunochemistry (Novocastra Laboratories, Newcastle upon Tyne, UK). The surgical specimens were stained for p53 (over)expression by immunohistochemistry using target unmasking fluid as described previously.¹² Prior to the study, immunocytochemistry and the polymerase chain reaction/restriction fragment

Results of p53 immunocytochemistry and PCR-RFLP analysis with subsequent ASO hybridisation for *K-ras* codon 12 mutations in cytology samples obtained from 17 patients with pancreatic carcinoma

Case No.	p53 Immunostaining		PCR mutation		ASO hybridisation		New amino acid
	Surgical	Cytological	Surgical	Cytological	Surgical	Cytological	
1	—	—*	—	—	—	—	
2	+	+	—	no DNA	—	—	
3	+	+	++	++	GAT	GAT	ASP
4	+	—*	++	++	GAT	GAT	ASP
5	+	—*	+	+	CGT	CGT	ARG
6	—	—	++	++	GAT	GAT	ASP
7	—	—	—	—	—	—	
8	+	+	—	—	—	—	
9	+	+	—	—	—	—	
10	—	—	+	++	GAT	GAT	ASP
11	+	—*	++	++	GTT	GTT	VAL
12	+	+	++	no DNA	GAT	—	ASP
13	+	—*	—	—	—	—	
14	—	—	+	++	GTT	GTT	VAL
15	+	+	++	++	GAT	GAT	ASP
16	+	—*	+	++	GTT	GTT	VAL
17	—	—	—	—	—	—	

* Cytology samples stained with Giemsa.
Mutant and wild-type on gel (+); only mutant band in RFLP (++)

Figure 2 Examples of *K-ras* mutation analysis on archival cytology specimens of patients with pancreatic cancer. **A:** RFLP analysis of mutant enriched PCR products. Lanes labelled as + were incubated with *Bst*NI after a second PCR amplification. The position of the *Bst*NI resistant (135 base pairs) and the cleaved (106 base pairs) products are indicated. **B:** Autoradiographs of ASO hybridisation performed with the oligonucleotide probe containing GTT at codon 12. Cases 11 and 16 are positive for this mutation on both cytology (left) and histology (right). HFL = wild-type (GGT) human cell DNA; SW480 = GTT mutation positive colon carcinoma cell line; GAT/CGT = control samples with a GAT or CGT mutation, respectively.

length polymorphism analysis (PCR-RFLP) were assessed and developed on the human colon cancer cell line SW480,¹²⁻¹⁴ with known *K-ras* and p53 mutations (fig 1). This also enabled us to determine the influence of Giemsa or PAP staining.

Based on the pattern of staining seen in the cell line, we developed a modified immunostaining procedure to optimise staining of the cytology specimens. Briefly, after dehydration in graded ethanol, the specimens were rinsed twice in distilled water and then fixed for 10 minutes in 4% paraformaldehyde in phosphate buffered saline (PBS). The specimens were then rinsed three times in distilled water and processed as described before for p53 immunocytochemistry.^{10 12 15}

After detection of p53 expression by immunocytochemistry and assessment of staining (nuclear positivity), the scrapings of the same glass slides were used for DNA isolation and PCR, as described previously.¹⁶

K-ras mutations were determined in both histological and cytological material by a combination of mutant enriched PCR-RFLP analysis and allele specific oligonucleotide (ASO) hybridisation.⁶ Briefly, this technique uses a mismatched 5' primer which generates a *Bst*NI restriction site at *K-ras* codon 12, such that digestion of the PCR product with *Bst*NI cleaves wild-type sequences and leaves amplified mutant alleles intact. Mutations are then verified and typed by ASO hybridisation.

In this study a cytology test was regarded as positive when either p53 immunocytochemistry or *K-ras* PCR-RFLP analysis with subsequent ASO hybridisation was positive. Efficacy was defined as the percentage of tumours correctly classified by the presence or absence of one of these molecular markers.¹⁰

Results

The results of the study are summarised in the table. Overexpression of p53 was detected in

six of 17 (35%) cytology specimens (fig 1). By contrast, p53 overexpression was observed in 11 (66%) of the 17 surgical specimens; in five cases p53 positivity observed on histology was not confirmed on cytology.

Resistance to *Bst*NI digestion was observed in 10 (60%) of the 17 cytology specimens (fig 2); scraping yielded insufficient DNA for PCR amplification in two cases. All cases with a positive result on PCR-RFLP analysis of the cytology specimens were also positive on histological analysis. In the five negative cytology cases PCR-RFLP analysis was also negative for the surgical specimens. Mutation was confirmed by ASO hybridisation in all positive cytology specimens. No discrepancies were found when ASO hybridisation was performed on the surgical and cytology specimens from the same patient. Mutations identified included guanine to adenine transitions (six cases), guanine to thymine transversions (three cases) and one guanine to cytosine transversion.

Based solely on either one of the cytological molecular markers, a diagnosis of pancreatic carcinoma would have been missed in four of 17 (24%) cases, resulting in an efficacy of 76%.

Four of the 17 cytology slides were read as negative or inconclusive on routine light microscopy; two of these were positive on either p53 immunocytochemistry or *K-ras* mutation analysis. This increased the diagnostic accuracy from 76 to 89%.

Discussion

K-ras and p53 gene mutations are believed to play a critical role in the development of invasive carcinoma of the pancreas. Detection of *K-ras* gene mutation provides an attractive molecular genetic probe for distinguishing between reactive and neoplastic changes in cytological samples. This is because *K-ras* mutations are present in most (80 to 90%) pancreatic carcinomas, because they occur early in

the development of pancreatic cancer, rendering early detection possible, and because they are limited to one codon and therefore detectable using a limited number of probes and primers.^{3,5-7} Mutations of the p53 tumour suppressor gene are similarly attractive because these mutations lead to stabilisation and therefore apparent overexpression of the p53 protein product, which can be readily detected using immunocytochemistry. Furthermore, p53 mutations occur exclusively in situ or invasive carcinoma of the pancreas.^{4,7,13}

The present study indicates that PCR followed by RFLP analysis and immunohistochemistry can be used to detect K-ras and p53 point mutations, respectively, in cytology specimens and suggests that these markers may be of use in the diagnosis of pancreatic cancer, confirming previous reports.¹⁷⁻²⁰ These techniques, when applied to archival tissue, were not without some technical problems. Of the 17 carcinomas, only six (35%) showed p53 overexpression on cytology and in five cases this result was not confirmed by histology. These five cases were stained with Giemsa which appears to interact adversely with the p53 immunostaining procedure. In the colon cancer cell line SW480 we were unable to achieve reliable p53 immunostaining following Giemsa staining, whereas immunocytochemistry performed on cytological specimens stained using the PAP method gave excellent results. In addition, one discordant sample (case 13) was a sampling error since no neoplastic tumour cells were detected in this specimen on routine cytological evaluation.

Technical procedures such as fixation are critical for optimal p53 immunocytochemistry and in that regard the retrospective nature of this study has important drawbacks. In prospective studies preparation of separate samples for p53 immunocytochemistry, which could then be used for DNA isolation and PCR, may be advisable.

K-ras mutations were detected in 10 of 17 (59%) cases. There was no discrepancy between the results obtained on histology and cytology suggesting that immunocytochemistry performed on cytological material even after routine fixation and staining has no adverse effects on subsequent PCR amplification and ASO hybridisation. The frequency distribution of the specific mutations—glycine to aspartic acid in 60%, glycine to valine in 30% and glycine to arginine in 10%—is in good agreement with the results obtained in our previous⁶ and in other studies.^{3,5} Interestingly, all positive results on PCR-RFLP analysis were confirmed by ASO hybridisation. This indicates that PCR-RFLP analysis is a sufficiently reliable methodology for the determination of the presence or absence of mutations when there is no need to specify the exact type of mutation present. As PCR-RFLP analysis is a relatively simple and straightforward technique, this would be the preferred methodology for routine screening of mutations in cytological samples or in pancreatic juice.^{17,18} ASO hybridisation can be

used for confirmation of the diagnosis in borderline cases.

K-ras or p53 mutations are indicative of malignancy. On combining these tumour markers, 13 of the 17 cases were positive, yielding 76% efficacy. As an adjunct to routine procedures this could certainly increase the diagnostic yield of pancreatic cytopathology. The rate of p53 overexpression and K-ras mutation in non-neoplastic pancreatic specimens needs to be explored in larger series of patients.

Two cases in this small study illustrate the potential use of the diagnostic application of p53 and K-ras assays as a supplemental technique in diagnostic cytology. In one case, in which the material was inconclusively diagnosed as "atypical cells suspicious for malignancy" (case 8), the demonstration of p53 overexpression confirmed malignancy. In the second case (case 14), the initial diagnosis of "atypical cells", either reactive or neoplastic, was complemented by the unequivocal presence of a K-ras mutation.

In summary, while there are important technical caveats, this study indicates that molecular and immunocytochemical techniques for detecting K-ras and p53 mutations are feasible and that detection of these molecular markers may be a valuable adjunct to diagnostic cytopathology.

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