Diagnosis of pancreatic lesions using fine needle aspiration cytology: Detection of K-ras point mutations using solid phase minisequencing

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Abstract

Aims—To improve the diagnostic value of fine needle aspiration biopsy of pancreatic lesions using a simple mutation detection method based on the polymerase chain reaction (PCR).

Methods—Fine needle aspirates from 21 suspected pancreatic lesions were analysed for K-ras codon 12 point mutations using solid phase minisequencing.

Results—A point mutation in codon 12 of the K-ras gene was detected in 14 of 17 cases of pancreatic carcinoma. No false positive results were recorded. The concordance of the result with routine cytology was 78%. All patients diagnosed as having malignant disease on cytology also had a K-ras point mutation. Additional information on the presence of malignancy was obtained using molecular genetic analysis in two cases.

Conclusions—PCR based minisequencing is a promising method for the analysis of cytological material. K-ras point mutation analysis was modified to enable it to be carried out in a clinical laboratory. Advantages of the method include its simplicity and speed. Adequate sampling guidance is important but analysis can be performed even with small amounts of cellular material.

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New technologies for enhancing the diagnostic yield of fine needle aspiration cytology have been reported recently.¹ Visualisation of tumours using radiological methods and analysis of immunological and genetic markers have improved greatly. The most promising methods for analysing small numbers of cells are those based on the polymerase chain reaction (PCR). Solid phase minisequencing, a single nucleotide primer extension method, has been used in the analysis of both acquired and hereditary point mutations.^{2,3} This method is precise, easy to use, and readily automated.

Some malignant disorders have distinctive molecular genetic characteristics. Point mutations of codon 12 of the K-ras gene occur in up to 90% of pancreatic adenocarcinomas, a feature which may be used in the diagnosis of these cancers.⁴ Alterations in the K-ras gene provide a direct approach to diagnosis and characterisation of a malignancy because their location and nature are well known and well

conserved. At present, most clinical laboratory methods used to analyse cancers at the molecular level have been modified from research laboratory protocols.^{5,6} The purpose of this study was to develop and evaluate simpler methods for performing clinical molecular genetic cytology and to compare its validity with cytological findings. Solid phase minisequencing was chosen because it provides a clear, numerical result and can be easily automated.

Methods

Biopsy specimens taken from 21 consecutive patients (11 women) with suspected pancreatic cancer were analysed. The mean age of the patients was 64 years (range 32 to 96 years). The patients had been admitted to the Clinic of Radiology at Helsinki University Central Hospital for needle biopsy during 1992 and 1993 because of suspected non-infectious pancreatic lesions. Thereafter, the patients were followed for at least one year. Biopsies were performed using ultrasound guided aspiration with 0.7–0.9 × 40–150 mm disposable needles under local anaesthesia. Each needle was attached to a 10 ml syringe in a hand-held biopsy extractor (Cameco, Enebyberg, Sweden). After the samples for routine diagnostic cytology were dispensed, the residual cells were injected into 1 ml physiological saline in a 1.5 ml Eppendorf tube. Routine cytological analysis was performed on alcohol fixed cells stained using the Papanicolaou method.

Each study sample was processed for storage within 45 minutes. The cells were centrifuged briefly at 10 000 rpm and the supernatant fluid discarded. The cells were stored in –70°C. The cell samples were prepared for PCR as described previously.^{3,7} The primers, one of which was biotinylated at the 5' end, used for nested PCR amplification of the region of interest are presented in table 1. PCR was performed using *Taq* DNA polymerase and amplification buffer from the same manufacturer (Promega, Madison, Wisconsin, USA). The reaction conditions were essentially as described before.³ Techne (Techne, Duxford, Cambridge, UK) or LKB (Pharmacia LKB Biotechnology, Bromma, Sweden) PCR thermocyclers were used. The temperature protocol was 95°C for one minute, 55°C for one minute, and 72°C for one minute.

For minisequencing analysis of the K-ras codon 12, PCR products amplified using

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Table 1 PCR and sequencing primers used in K-ras codon 12 minisequencing analysis

Primer	Sequence
PCR 5' primer	AGG CCT GCT GAA AAT GAC TG
PCR 3' primer	AAG ATT TAC CTC TAT TGT TGG
Nested 5' primer	GAC TGA ATA TAA ACT TGT GG
Nested 3' primer (biotinylated)	CCT CTA TTG TTG GAT CAT AT
Codon 12:1 5' sequencing primer	AAC TTG TGG TAG TTG GAG CT
Codon 12:2 5' sequencing primer	ACT TGT GGT AGT TGG AGC TT

biotinylated primers were bound to strept-
avidin coated microtitration plates. The PCR
products were then denaturated and the
minisequencing reaction performed as
described before (figure).⁸ The reaction
proceeded for 60 minutes at 37°C. The unbound
reaction components were then washed away
using an automatic washer (Delfia Platewash,
Wallac, Turku, Finland). The microtitration
plates used (ScintiStrips, Wallac) were fabri-
cated from scintillating plastic and only
needed to be air dried for 30 minutes before
being inserted into a microtitration plate for-
mat scintillation counter (Wallac Microbeta,
Wallac). In minisequencing analysis point
mutations are detected by comparing the
incorporated radioactivities (counts per
minute) of the normal and aberrant
nucleotide (figure), and are expressed as a
ratio of incorporation of normal and abnormal
nucleotides (R value). When screening for
mutations a mixture containing all the
aberrant nucleotides in one reaction was used.
The positive findings were confirmed using
specific single nucleotide primer extension
reactions. The results are presented in table 2.
Both positive (SW 116 cell line, ATCC,
Rockville, Maryland, USA) and negative
(DNA from healthy subjects) internal controls
were included in each series. A +3 SD
increase from the control value was indicative
of a positive result. Two parallel minisequenc-

A flow chart of the minisequencing assay. The
minisequencing procedure was performed in 96 well
microtitration plates. The plates were coated with
streptavidin and served as the solid phase in the assay. The
single nucleotide primer extension reactions for the normal
and mutant nucleotides were performed in separate wells.
The amount of mutated compared with normal PCR
product was indicated by the incorporated radioactivity in
the corresponding wells of the microtitration plate. The
ratio of these radioactivities was defined as the R value.
For example, if the sample only contains normal DNA the
activity in the G reaction well is about 3000 counts per
minute. The corresponding activity in the A, C, and T
reactions is essentially background—for example, 50 counts
per minute. The abnormal count rate was divided by the
normal one. Thus, the R value of the example is 0.17.

Table 2 Clinical characteristics and results of K-ras mutation analysis in patients with pancreatic lesions

Case No	Cytological diagnosis	Final diagnosis	Minisequencing results	
			R values* 12:1	R value 12:2
1	Cyst	Pancreatic cancer	0.059	0.086
2	Inflammation	Pancreatic pseudocyst	0.049	0.059
3	Inflammation	Pancreatic cancer	0.093	0.078
4	Suspected carcinoma	Pancreatic cancer	0.077	2.082
5	Carcinoma	Pancreatic cancer	0.052	0.426
6	None	Sclerosing cholangitis	0.015	0.029
7	Carcinoma	Pancreatic cancer	0.159	0.034
8	Carcinoma	Pancreatic cancer	0.039	3.449
9	Normal	Pancreatic cancer	0.02	0.501
10	Carcinoma	Pancreatic cancer	0.039	3.754
11	Carcinoma	Pancreatic cancer	0.013	0.461
12	Carcinoma	Pancreatic cancer	0.31	0.057
13	Carcinoma	Pancreatic cancer	0.026	0.242
14	Carcinoma	Pancreatic cancer	0.026	1.866
15	Carcinoma	Pancreatic cancer	0.047	8.979
16	Carcinoma	Pancreatic cancer	0.026	0.295
17	None	Benign cystadenoma	0.022	0.035
18	Inadequate sample	Pancreatic cancer	0.021	0.033
19	Carcinoma	Pancreatic cancer	0.033	0.461
20	Carcinoma	Pancreatic cancer	0.027	0.353
21	None	Intestinal lymphoma	0.028	0.039
		Control mean	0.039	0.045
		Control SD	0.02	0.02
		Mean + 3 SD	0.099	0.105

The R values of samples with mutations are in italics and the two borderline values are in bold. The R value was obtained by dividing the reactivity (counts per minute) of mutant nucleotides by the radioactivity (counts per minute) of the normal nucleotide. The R value of the control group was determined by analysing five healthy subjects, one patient with a lymphoma who was not expected to have a K-ras mutation, and three inflammatory cell samples from bronchoalveolar lavage fluid specimens from patients with lung infections.

ing reactions were performed for each nucleo-
tide and sample, and served as additional
controls for potential technical faults.

Results

Minisequencing revealed K-ras codon 12
mutations in 14 of 21 cases. Of these muta-
tions, 12 occurred in the second nucleotide of
the codon and two at the first. This finding
agrees with earlier reports of the distribution
of K-ras mutations in pancreatic cancer.⁴
When minisequencing data were compared
with the primary cytopathological reports, all
12 patients with pancreatic adenocarcinoma
on cytology had point mutations in the K-ras
gene. Two other patients, one cytologically
normal, the other with suspected pancreatic
cancer, also had point mutations. Clinical
data obtained at follow up and histological
biopsy reports confirmed their final diagnosis
as pancreatic adenocarcinoma. Two of the 21
patients were diagnosed as having pancreatic
inflammation and a cyst. A third patient had
inadequate sample. They were normal on the
point mutation analysis, but later diagnosed

as having carcinomas by other means (histology and clinical examination). One patient had an intestinal lymphoma diagnosed on cytology and histology. His sample was negative on *K-ras* point mutation analysis.

Two patients (cases 1 and 3) had borderline R values (+2 SD above normal). They did not show significant aberrant incorporation on single nucleotide incorporation analysis and thus were not regarded as having point mutations. Both of these patients had pancreatic adenocarcinomas.

Discussion

We studied the effect of fast and reliable point mutation analysis for the detection of malignancy using fine needle aspiration biopsy specimens of pancreatic lesions. Our results show that the specificity and sensitivity of *K-ras* point mutation analysis of ultrasound guided pancreatic fine needle biopsy specimens are as good as those of standard microscopic cytology. *K-ras* point mutations occur in up to 90% of pancreatic adenocarcinomas.⁹ In this study all carcinomas diagnosed on cytology also had a point mutation.

Samples with substantial amounts of necrosis or inflammatory changes are more likely to give false negative results in PCR based tests. This may be because of a lack of malignant cells, dense inflammatory cell background, or the presence of bacterial DNA digesting enzymes. Our observations confirm that a good sampling technique and proper needle localisation are prerequisites for successful fine needle biopsy molecular cytology. There is a 10–40% false negative rate in the diagnosis of pancreatic malignancies with guided biopsy.¹⁰ The pancreatic mass comprises both malignant cells and secondary inflammatory areas which explains the inevitable false negative results.

On the other hand, samples with good morphology and a large number of apparently normal cells are good candidates for amplification using PCR. The efficacy of PCR in the surveillance of residual acute leukaemic disease has been reported before.¹¹ In this study the sample morphologically diagnosed as normal pancreatic tissue, but containing malignant cells on minisequencing, provides evidence that PCR amplification is a highly sensitive method for detecting malignant cells against a normal cell background. The two cancer patients with borderline R values show that the sensitivity of this method for detecting mutations can be increased even further. In these cases we could not confirm the pres-

ence of a point mutation using specific nucleotide incorporation analysis. This may be because only one nucleotide is present in the specific reaction. In the screening reaction three different nucleotides are available, enabling the polymerase to elongate the chain following mutation. The extra nucleotides potentiate the search for mutations. Thus, the reaction may be more sensitive to very small amounts of mutated alleles if a mixture containing all three possible mutant nucleotides is used. Magnetic microspheres can also be used as a solid phase, increasing the sensitivity of the assay.^{3,12}

The methods used in this study are relatively easy to use and maintain, and can be modified and adapted to non-radioactive detection systems. The protocols can be standardised and the procedure is almost identical for any known point mutation, facilitating the analysis of multiple point mutations in a single minisequencing series.

In conclusion, PCR based analysis of ultrasound guided fine needle biopsy material is a promising method for enhancing diagnostic accuracy in central cytopathology laboratories. The basic principles of diagnostic cytology, however, must also be taken into account.

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