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

J Ihalainen, M Taavitsainen, T Salmivaara, A Palotie

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.

New technologies for enhancing the diagnostic yield of fine needle aspiration cytology have been reported recently.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{5} 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). 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 (Camco, 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.\textsuperscript{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.\textsuperscript{3} PCR products amplified using Taq DNA polymerase 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
Table 1 PCR and sequencing primers used in K-ras codon 12 minisequencing analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR 5' primer</td>
<td>AGG CCT GCT GAA AAT GAC TG</td>
</tr>
<tr>
<td>PCR 3' primer</td>
<td>AAG ATT TAC CTC TGT TGG</td>
</tr>
<tr>
<td>Nested 5' primer</td>
<td>GAC TGA ATA TAA ACT TGT GG</td>
</tr>
<tr>
<td>Nested 3' primer</td>
<td>CCT CTA TGG TGG CAT CAT AT</td>
</tr>
<tr>
<td>Codon 12:1 5' sequencing primer</td>
<td>AAC TGG TGG TAG TGG AGG TT</td>
</tr>
<tr>
<td>Codon 12:2 5' sequencing primer</td>
<td>ACT TGT GGT AGT TGG AGC TT</td>
</tr>
</tbody>
</table>

Biotinylated primers were bound to streptavidin coated microtitration plates. The PCR products were then denatured and the minisequencing reaction performed as described before (figure). 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 fabricated from scintillating plastic and only needed to be air dried for 30 minutes before being inserted into a microtitration plate format 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 minisequencing reactions were performed for each nucleotide and sample, and served as additional controls for potential technical faults.

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

<table>
<thead>
<tr>
<th>Case No</th>
<th>Cytological diagnosis</th>
<th>Final diagnosis</th>
<th>Minisequencing results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R value * 12:1</td>
</tr>
<tr>
<td>1</td>
<td>Cyst</td>
<td>Pancreatic cancer</td>
<td>0.059</td>
</tr>
<tr>
<td>2</td>
<td>Inflammation</td>
<td>Pancreatic pseudocyst</td>
<td>0.049</td>
</tr>
<tr>
<td>3</td>
<td>Inflammation</td>
<td>Pancreatic cancer</td>
<td>0.093</td>
</tr>
<tr>
<td>4</td>
<td>Suspected carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.077</td>
</tr>
<tr>
<td>5</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.052</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>Sclerosing cholangitis</td>
<td>0.015</td>
</tr>
<tr>
<td>7</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.159</td>
</tr>
<tr>
<td>8</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.039</td>
</tr>
<tr>
<td>9</td>
<td>Normal</td>
<td>Pancreatic cancer</td>
<td>0.02</td>
</tr>
<tr>
<td>10</td>
<td>Normal</td>
<td>Pancreatic cancer</td>
<td>0.039</td>
</tr>
<tr>
<td>11</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.013</td>
</tr>
<tr>
<td>12</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.021</td>
</tr>
<tr>
<td>13</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.026</td>
</tr>
<tr>
<td>14</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.026</td>
</tr>
<tr>
<td>15</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.047</td>
</tr>
<tr>
<td>16</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.026</td>
</tr>
<tr>
<td>17</td>
<td>None</td>
<td>Benign cystadenoma</td>
<td>0.022</td>
</tr>
<tr>
<td>18</td>
<td>Inadequate sample</td>
<td>Pancreatic cancer</td>
<td>0.031</td>
</tr>
<tr>
<td>19</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.033</td>
</tr>
<tr>
<td>20</td>
<td>Carcinoma</td>
<td>Pancreatic cancer</td>
<td>0.027</td>
</tr>
<tr>
<td>21</td>
<td>None</td>
<td>Intestinal lymphoma</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control mean</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control SD</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean + 3 SD</td>
<td>0.099</td>
</tr>
</tbody>
</table>

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.

Results

Minisequencing revealed K-ras codon 12 mutations in 14 of 21 cases. Of these mutations, 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 nega-
tive 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 pancre-
atic adenocarcinomas.

Discussion
We studied the effect of fast and reliable point mutation analysis for the detection of malign-
nancy 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 micro-
scopic 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 necro-
sis 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 nega-
tive results.

On the other hand, samples with good mor-
phology and a large number of apparently
normal cells are good candidates for amplifi-
cation using PCR. The efficacy of PCR in
the surveillance of residual acute leukemic dis-
ease has been reported before.11 In this study
the sample morphologically diagnosed as normal
pancreatic tissue, but containing malign-
ant 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 detect-
ing 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,
allowing 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 con-
taining all three possible mutant nucleotides is
used. Magnetic microspheres can also be used
as a solid phase, increasing the sensitivity of
the assay.12

The methods used in this study are rela-
tively easy to use and maintain, and can be
modified and adapted to non-radioactive
detection systems. The protocols can be stan-
dardised 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 ultra-
sound guided fine needle biopsy material is a
promising method for enhancing diagnostic
accuracy in central cytopathology laborato-
ries. The basic principles of diagnostic
cytology, however, must also be taken into
account.

1 Bylcy JD. Update on special techniques in routine
2 Syvänen A-C, Asot-Anriätä K, Hatur L, Kontula K,
Söderlund H. A primer-guided nucleotide incorporation
assay in the genotyping of apolipoprotein E. Genomics
3 Syvänen A-C, Söderlund H, Laaksonen E, Bengtsson M,
Turenne M, Paleit A. N-Ras gene mutations in acute
myeloid leukemia: accurate detection by solid-phase
4 Bos JL. Ras oncogenes in human cancer: a review. Cancer
5 Ranta-Aalhä M, Hwangfeldt-Purisäkitä K. Denaturing
gradient gel electrophoresis (DGGE) assay for K-ras and
N-ras genes: detection of K-ras point mutations in human
6 Urban T, Ricci S, Grave J-J, Lacave B, Boudigene F,
Breitmayer F, et al. Detection of c-Ki-ras mutation by
PCR/RFLP analysis and diagnosis of pancreatic ade-
7 Syvänen A-C, Vonk E, Manninen T, Bengtsson M,
Söderlund H, Aita P, et al. Convenient and quantitative
determination of the frequency of a mutant allele using
solid-phase minisequencing: application to aspartyl-
glu-
8 Ihalainen J, Sittari H, Laine S, Syvänen A-C, Paleit A.
Towards automatic detection of point mutations: use of
scintillating microtitration plates in solid phase minise-
9 Hirtan RH, Van Mantefield AMD, Offerhaus GJA,
K-ras oncogene activation in adenocarcinomas of the
10 Mueller PR. Pancreatic biopsy: Striving for excellence.
11 Negrin RS, Blume KG. The use of the polymerease
chain reaction for the detection of minimal residual
12 Paleit A, Syvänen A-C. Development of molecular
genetic methods for monitoring myeloid malignancies.