Use of microsatellite marker loss of heterozygosity in accurate diagnosis of pancreaticobiliary malignancy from brush cytology samples

A Khalid, R Pal, E Sasatomi, P Swalsky, A Slivka, D Whitcomb, S Finkelstein


Early and accurate diagnosis of pancreaticobiliary malignancy offers the best chance of a surgical cure while avoiding unnecessary major surgery in patients with benign disease. In many patients the first sign of a pancreaticobiliary cancer is a stricture of the bile duct. Currently, the diagnostic process includes an endoscopic retrograde cholangiography with brushings of the stricture to obtain cells used for cytological examination. The diagnosis of malignancy is based on cell morphology alone. However, a definitive diagnosis is impossible in many cases due to low cellularity, morphological changes induced by inflammation or necrosis, or technical variables in sample preparation associated with drying or cellular degradation during processing. Furthermore, a morphological diagnosis is subjective and observer dependent. Together, these factors result in a diagnostic test with low sensitivity (<60%).1–5

The altered morphology of malignant cells reflects underlying genetic changes. The progressive morphological changes seen in the development of pancreatic ductal adenocarcinoma from normal cells has recently been modelled in the pancreatic intra ductal neoplasia (PanIN) system.2 The strength of this system includes standardised pathological criteria for each progressive stage in tumour development linked with the underlying genetic aberrations.6–14 K-ras codon 12 mutations, for example, represent one of the earliest genetic changes in the development of pancreatic cancer15,16 but there remains debate as to the exact frequency in bile duct cancer.17,18 Sequential inactivation of tumour suppressor gene is also seen. This process can occur through a variety of processes, including gene mutation, hypermethylation, or loss of a chromosome or chromosomal segment containing the tumour suppressor gene. Any combination of these events will lead to loss of tumour suppressor gene activity. Tumour suppressor and related genes commonly lost in pancreaticobiliary cancers include TP53, p21, p16Ink4a/CDKN2A and DPC4/SMAD4, p53, and APC.19–39

We hypothesised that representative cells derived from malignant strictures would manifest a high level of accumulated mutational damage reflective of an underlying tumour, and that similar alterations would not be seen in cells reacting to an inflammatory process. As mutational screening of all relevant tumour suppressor genes from a few cytological cells is currently impossible in most diagnostic laboratories, and as a major cause of tumour suppressor gene inactivation is due to chromosomal loss (loss of heterozygosity, LOH) we further hypothesised that detection of LOH from microsatellite markers closely linked to key genes would serve as an excellent surrogate marker for gene inactivation. Recognising that malignant strictures could be derived from pancreatic or biliary epithelial origin and that various tumour suppressor genes are lost at different stages of tumorigenesis and in only a subset of cancers requires that a broad panel of loci must be considered. The purpose of the current study was...
to construct such a panel of LOH markers together with k-ras codon 12 activation mutation detection and to test this panel on brush cytology cells compared with surgical resected tumours, using normal cells from the same patient as internal controls.

**MATERIALS AND METHODS**

Twenty six patients with surgical (n = 25) or a long term disease free follow up (n = 1) were selected. Seventeen patients had surgically proven cancer (pancreatic adenocarcinoma (n = 6), cholangiocarcinoma (n = 11)). Nine patients had an inflammatory process of which eight underwent surgical resection. Brush cytology was available for all patients which was recorded as malignant (n = 8), indeterminate (n = 10), and negative for malignancy (n = 8). Of the 10 patients with inconclusive cytology, one had a benign process and eight cases with a benign aetiology were reported “negative for malignant cells”. The cytopathological criteria for malignancy included nuclear enlargement, pleomorphism and eight cases with a benign aetiology were reported “negative for malignant cells”. The cytopathological criteria for malignancy included nuclear enlargement, pleomorphism (minimum of 3–4-fold variation in nuclear size), elevated N/C ratio, nuclear membrane irregularity, and coarse chromatin. Cases diagnosed as inconclusive fulfilled most but not all of the criteria for malignancy. The study was reviewed and approved by the University of Pittsburgh Medical Center, Institutional Review Board.

Cellular material from cytopathology and surgical pathology slides was collected by microdissection for LOH analysis and k-ras-2 point mutation determination. For cytology specimens, clusters of abnormal appearing cells were identified and marked on representative alcohol fixed Papanicolaou stained slides. Areas of interest were manually microdissected from the slides and placed in 25 μl of dilute Tris buffer, pH 7.5. For surgical pathology slides, normal biliary tissue (negative control) and neoplastic areas were manually microdissected from all malignant (17) and two benign cases. Normal tissue samples were run to determine normal microsatellite heterozygosity as well as to serve as an internal negative control for mutational damage.

In some cases, only a small number of abnormal appearing cells were identified on brush cytology slides. Therefore, two different approaches were used for LOH analysis and the results compared. The first approach, termed collective microdissection (CA), involved combining separate aggregates of abnormal appearing cell clusters into a single storage sample of sufficient cellular content to allow for direct polymerase chain amplification reaction (PCR). A minimum of approximately 1000 cells was necessary for sufficient genomic DNA for this type of direct analysis. The CA approach thus produced an averaging of mutational change among the aggregated microdissected cells. The second approach, termed whole genome amplification (WGA), consisted of microdissection of discrete clusters of 50–100 cells representing individual cytological lesions. In order to obtain sufficient DNA substrate for broad panel genotyping, WGA was performed prior to individual marker PCR amplification and analysis. Two to three separate WGA were carried out on individual cases. The WGA approach could be applied in situations of scant cellularity where inadequate cells were present for the CA approach. Also, the WGA method was considered theoretically capable of providing data on intratumoral heterogeneity and cancer clonal expansion so that the different microdissected samples could be compared with each other to gain additional insight into tumorigenesis and cancer biology. WGA, however, introduced an intermediary step that could introduce artefacts into final mutational profiling and therefore was directly compared with the results of CA. WGA was performed as previously reported using the degenerate oligonucleotide primed-PCR technique.

**Table 1** Tumour suppressor genes (with associated markers) and k-ras-2 gene with chromosomal location and mutation type

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Short arm marker</th>
<th>Long arm marker</th>
<th>Mutation type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoblastoma interacting zincfinger (RIZ)</td>
<td>1p36–1p34</td>
<td>D1S407</td>
<td>MYCL</td>
<td>Deletion</td>
</tr>
<tr>
<td>von Hippel-Lindau (VHL)</td>
<td>3p26–3p25</td>
<td>D3S539</td>
<td>D3S2303</td>
<td>Deletion</td>
</tr>
<tr>
<td>Adenomatous polyposis coli (APC)</td>
<td>5q23–5q23</td>
<td>D5S592</td>
<td>DSS615</td>
<td>Deletion</td>
</tr>
<tr>
<td>CDKN2A/p16</td>
<td>9p21–9p23</td>
<td>D9S251</td>
<td>D9S254</td>
<td>Deletion</td>
</tr>
<tr>
<td>Phosphatase and Ten sin homologue deleted on chromosome Ten (PTEN)</td>
<td>10q22–10q23</td>
<td>D10S520</td>
<td>D10S1173</td>
<td>Deletion</td>
</tr>
<tr>
<td>P53</td>
<td>17p13–17p13</td>
<td>D17S974</td>
<td>D17S1289</td>
<td>Point mutation</td>
</tr>
</tbody>
</table>

Aliquots (1 μl) of CA DNA or WGA PCR products were used in the PCR for a broad panel of microsatellite markers potentially commonly involved in human pancreatic and biliary carcinogenesis. Tumour suppressor gene LOH was determined by analysis of tightly linked informative polymorphic microsatellites. The tumour suppressor genes selected are given in table 1. Use of two markers within each locus was used to increase the likelihood that at least one of the markers would be polymorphic within a subject, and thus informative for LOH analysis.

A microsatellite is a region of genomic DNA with a string of 1–4 bases that are repeated over a short distance. The number of repeats and a locus is often variable between alleles so that each chromosome can be identified and traced. Since tens of thousands of microsatellites with heterogeneous sizes span the human genome it is possible to choose highly heterogeneous microsatellites as chromosomal markers at loci that immediately flank tumour suppressor genes or other genes of interest. LOH (for example, either the shorter or longer microsatellite is missing) suggests that one of the two chromosomal arms has been lost. The implication is that a mutation of the tumour suppressor gene on the opposite chromosome occurred because the clone of tumour cells with this combination of LOH and a tumour suppressor gene mutation would provide a growth advantage.

PCR amplification was designed to generate amplions of less than 200 base pairs long using synthetic oligonucleotide primers flanking each microsatellite. Oligonucleotide primers were created with 5’ fluorescent moieties (FAM, HEX, NED) suitable for automated fragment analysis. PCR products were analysed by capillary electrophoresis on an ABI 3100 according to manufacturer’s instructions (Applied Biosystems, Foster City, California, USA). Allele peak heights and lengths were used to define the presence or absence of allelic imbalance (that is, LOH) for a given sample. Allelic imbalance was reported when the ratio of polymorphic allelic bands for a particular marker was below 0.5 or above 2.0. In
addition, the deleted allele was designated as either “B” or “T” depending on whether the bottom (longer length) or top (shorter length) microsatellite allele was diminished compared with the subject’s normal DNA profile. This was important as the presence of the identical deleted allele in different microdissection targets supported the existence of the same deletion in all affected target sites. Similarly, it was possible to identify two separate mutations of the same genomic region in different topographic tissue samples when deleted alleles were shown to be discordant. A locus was determined to be concordant when the same allele was lost (for example, LOH B in the cytology specimen and the surgical specimen).

In addition to allelic loss analysis, DNA sequencing of k-ras-2 exon 1 PCR amplified DNA was used to search for and characterise point mutations in codons 12 and 13. Overall genotyping analysis thus represented a combination of point mutational and allelic loss damage (table 1).

Statistical analysis
The fractional mutation rate (FMR), defined as the number of mutated markers (k-ras-2 point mutation with or without significant allelic imbalance) divided by the total number of informative microsatellite markers plus 1 for k-ras-2 status, was used as a measure of overall mutation accumulation. Recognising that each patient possessed his/her own unique panel of informative polymorphic microsatellite markers, the FMR served as a means of comparing patients to each other with respect to the extent of cumulative acquired mutational damage. Mean number of mutations between the positive and inconclusive cytology was compared using a two independent sample t test. Comparison of mutations between malignant and benign samples utilised the non-parametric two sample Mann-Whitney U test. Furthermore, slight variations in the mutational profile often, however, yielded multiple losses, the FMR ranging from 0.3 to 0.7 (average 0.45) for positive and from 0.3 to 0.7 (average 0.38) for indeterminate samples, without significant difference. The presence of k-ras mutations was associated with pancreatic cancer (5/6) and not cholangiocarcinoma (0/11) and this difference was significant (p<0.001).

The pattern of allelic loss and k-ras-2 point mutational damage in all microdissected tissue samples closely correlated with the corresponding tissue specimens. In cases where a k-ras-2 point mutation was identified in the resected tissue samples, the identical k-ras-2 mutated alteration was present in the cytology specimens using both the CA and WGA approach. In some cases, the mutational profile defined by cytological specimens was a perfect match to that obtained from microdissected tissue samples. More often, however, slight variations in the mutational profile were evident between the cytological and histological

<table>
<thead>
<tr>
<th>Case No</th>
<th>Pathology</th>
<th>Cytology</th>
<th>Path-LOH*</th>
<th>Cyto-LOH†</th>
<th>Normal-LOH‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pancreatic cancer</td>
<td>Positive</td>
<td>k-ras, RIZ, VHL, PTEN, P53</td>
<td>k-ras, RIZ, VHL, PTEN, P53</td>
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<td>2</td>
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<tr>
<td>3</td>
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<td>RIZ, P16</td>
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<td>k-ras, VHL, APC, P16, PTEN</td>
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</tbody>
</table>

*Path-LOH, loss of heterozygosity (LOH) in the surgically resected tumour, following microdissection.
†Cyto-LOH, LOH in cells from the cytology specimen.
‡Normal-LOH, LOH in normal tissue adjacent to the tumour.
material. These difference were interpreted as minor muta-
tional profile variations from intratumoral heterogeneity,
given that the specimens were taken from different parts of
the tumour within each case.27 28 Two examples of micro-
dissection genotyping from the series are shown in tables 3
and 4. The patient displayed in table 3 manifested a perfect
concordance of mutational change between cytological
samples obtained by both the CA method and the WGA
approach. Genotyping results from the patient shown in
table 4 revealed a minor degree of discordance affecting two
markers from six that exhibited mutational change. Despite
these slight differences, the majority of mutational change
was discordant between the different samples of tumour.

All cases characterised as suspicious or atypical for
malignancy by cytomorphology alone could be shown to
manifest a profile of accumulated mutational damage
equivalent to that seen in proven pancreaticobiliary cancer.
Irrespective of the reasons for indeterminate status,
the resulting profile of accumulated mutated markers was
sufficiently precise to afford firm correlation with ultimate
malignancy, as established in tissue specimens based on both
cellular morphology and cumulative mutational profile.

**DISCUSSION**

Cytological diagnosis of pancreaticobiliary cancer can be
challenging, especially with a high rate of indeterminate
diagnoses using brush cytology. Molecular approaches, such
as the one described here, provide an independent determi-
nation of the presence and extent of mutational damage that
underlie malignant transformation.49 50 The sensitivity,
specificity, and accuracy of our technique are 100%.

Two methods were used to collect cytological samples for
molecular analysis. The collective assembly (CA) approach
enabled direct application of genotyping to aggregated
representative cells and thus closely paralleled the tissue
based mutational analysis. The CA method has two impor-
tant drawbacks. The first is the requirement for relatively
abundant cellular material as broad panel mutational geno-
typing demands that the sample be subdivided for individual
mutational analyses. Abundance of cellular material is one of
the limitations in standard morphological analysis of cytology
specimens which was the reason for using an alternative
molecular approach for diagnostic assistance. The second
drawback of the CA method is the averaging between
different cellular clusters that are aggregated together as

<table>
<thead>
<tr>
<th>Gene</th>
<th>Non-neoplastic tissue</th>
<th>Positive cytology (CA)*</th>
<th>Positive cytology (WGA)</th>
<th>Positive cytology (WGA)</th>
<th>Histology tumour</th>
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</thead>
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<tr>
<td>RIZ</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
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<td>I</td>
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<td>LOH T</td>
<td>LOH T</td>
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<td>I</td>
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<td>NI</td>
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<td>NI</td>
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<td>P53</td>
<td>I</td>
<td>LOH B</td>
<td>LOH B</td>
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</tr>
</tbody>
</table>

*CA, collective assembly in which multiple discrete clusters of target cells are aggregated together into one common
pool from which aliquots are taken for broad panel mutational genotyping.

†WGA, whole genome amplification in which a single cluster of cells is first subject to general amplification using
random oligonucleotide primers.

NI, non-informative marker status (see materials and methods for further details); I, informative marker.
part of one sample that then is subdivided for broad panel molecular genotyping. This would have the effect of obscuring intratumoral mutational heterogeneity while favouring the detection of those alterations that arose early in tumorigenesis and are present throughout the majority of cancer cells.

The whole genome amplification technique (WGA) for analysing individual cytology cell clusters does not suffer from these drawbacks but itself could suffer from the problem of artefactual allelic imbalance introduced during the pre-genotyping amplification step. This important drawback is well recognised and must be carefully evaluated when the technique is used. Our approach was to output replicate analyses on both positive and negative results for each patient and to require that all such testing be shown to be consistent. Thus WGA appears to be useful for LOH analysis and point mutation analysis.

Although specific mutational damage can occur in reactive states of cellular proliferation (for example, k-ras-2 point mutations in chronic pancreatitis without evidence of malignancy), most malignancies, including pancreatic cancer, manifest abundant somatically acquired DNA mutational alterations in keeping with their neoplastic phenotype. In most systems, the level of accumulated mutations is significantly higher in frank cancer than in precancerous lesions. It is reasonable to contend that objective thresholds for accumulated DNA mutational damage can be formulated that discriminate between non-neoplastic reactive states versus malignancy with a high degree of confidence. A further insight from our results is the lack of k-ras mutations in biliary malignancy compared with pancreatic adenocarcinoma, thus patterns of mutation accrued in biliary strictures may help predict the origin of the underlying neoplasm.

In conclusion, a microdissection genotyping approach organised in series to follow cytological analysis can provide valuable information without jeopardising morphological interpretation. Commonly deleted chromosomal regions harbouring potential tumour suppressor genes can be detected by loss of heterozygosity (LOH) analysis using PCR amplification of polymorphic microsatellite repeats in tumour and matched normal DNA. When LOH analysis is extended to multiple chromosomal arms, a distinct allelotype is generated reflecting the malignant status of the corresponding cells and providing a potential independent and objective marker of malignancy. The overwhelming concordance in allelic loss and k-ras-2 point mutational change provides validation that molecular profiling of cytological specimens reflects an accurate and detailed picture of mutation acquisition unique to an individual patient’s tumour. The presence, extent, and pattern of acquired mutation damage in selected cell populations of endoscopic brush cytology provide meaningful discriminatory information for the improved diagnosis of pancreaticobiliary cancer. We recommend and foresee the application of such molecular techniques to reach a definitive diagnosis in the setting of inconclusive brush cytology. This will potentially avoid further invasive tests in patients with biliary strictures and expedite appropriate management.

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REFERENCES
Molecular pancreaticobiliary allelotyping


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Gut 2004 53: 1860-1865
doi: 10.1136/gut.2004.039784

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