PANCREAS AND BILIARY

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

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Background: Brush cytology of biliary strictures to diagnose pancreaticobiliary malignancy suffers from poor sensitivity.

Aim: To improve the diagnostic yield of pancreaticobiliary brush cytology through analysis of tumour suppressor gene linked microsatellite marker loss of heterozygosity (LOH) and k-ras codon 12 mutation detection.

Methods: Twenty six patients with biliary strictures underwent endoscopic retrograde cholangiography with brush cytology. A panel of 12 polymorphic microsatellite markers linked to six tumour suppressor genes was developed. Genomic DNA from cell clusters acquired from brush cytology specimens and microdissected surgical malignant and normal tissue underwent polymerase chain amplification reaction (PCR). PCR products were compared for LOH and k-ras codon 12 mutations.

Results: Seventeen patients were confirmed to have pancreaticobiliary adenocarcinoma. Nine patients had benign strictures (eight proven surgically, one by follow up). Cytomorphological interpretation was positive for malignancy (n=8), indeterminate (n=10), and negative for malignancy (n=8). Selected malignant appearing cytological cell clusters and microdissected histological samples from cancer showed abundant LOH characteristic of malignancy while brushings from nine cases without cancer carried no LOH (p<0.001). LOH and k-ras mutations profile of the cytological specimens was almost always concordant with the tissue samples. Presence of k-ras mutation predicted malignancy of pancreatic origin (p<0.001).

Conclusion: LOH and k-ras codon 12 mutation analysis of PCR amplified DNA from biliary brush cytology discriminates reactive from malignant cells, with 100% sensitivity, specificity, and accuracy. Minor variations in LOH in brushings and in different sites within the same tumour likely reflect intratumoral mutational heterogeneity during clonal expansion of pre- and neoplastic lineages.

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 intraductal neoplasia (PanIN) system.⁶ The strength of this system includes standardised pathological criteria for each progressive stage in tumour development linked with the underlying genetic aberrations.^{7–14} K-ras codon 12 mutations, for example, represent one of the earliest genetic changes in the development of pancreatic cancer^{15 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

Abbreviations: LOH, loss of heterozygosity; WGA, whole genome amplification; CA, collective assembly; FMR, fractional mutation rate; PCR, polymerase chain amplification reaction

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

Gene	Locus	Short arm marker	Long arm marker	Mutation type
Retinoblastoma interacting zincfinger (RIZ)	1p36-1p34	D1S407	MYCL	Deletion
von Hippel-Lindau (VHL)	3p26-3p25	D3S1539	D3S2303	Deletion
Adenomatous polyposis coli (APC)	5q23-5q23	D5S592	D5S615	Deletion
CDKN2A/p16	9p21-9p23	D9S251	D9S254	Deletion
Phosphatase and Ten sin homologue deleted on chromosome Ten (PTEN)	10q23-10q23	D10S520	D10S1173	Deletion
P53	17p13-17p13	D17S974	D17S1289	Deletion
k-ras	12p12			Point mutation

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 (minimum of 3–4-fold variation in nuclear size), elevated N/C ratio, nuclear membrane irregularity, and coarse chromatin.⁴⁰ 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 assembly (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.^{41–42} 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.^{42–43}

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 amplicons 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 2.0.⁴⁴ 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^{45, 46} 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 because of unequal variances. The χ^2 test was used to contrast specific mutational patterns in neoplastic tissue of pancreatic versus biliary origin.

RESULTS

Microsatellite polymorphic profiles were determined in normal tissue from all patients. In all patients with cancer and two with no cancer, genotyping data were available on a non-neoplastic sample from the subsequent tissue resection. Normal appearing cellular material from the brush cytology was used in the remaining patients. None of the non-neoplastic tissue targets showed evidence of allelic loss in multiple sampling, as defined by threshold criteria (see materials and methods). Data regarding the presence or absence of k-ras-2 point mutational changes were available for all patients.

All 17 patients with cancer in resected tissue specimens had multiple loci with LOH compared with their own normal tissue, with or without k-ras-2 point mutational change, yielding a sensitivity of 100%. Using tissue specimens of resected cancer, FMR ranged from 0.30 to 0.85. There was no LOH or k-ras-2 point mutations in any of the microdissected samples obtained from the nine subjects without cancer ($p < 0.001$) (table 2).

Mutational analysis of cytological specimens from cancer 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 cytology samples closely correlated with the corresponding tissue specimens. In all 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 2 Detailed information on all cases, including tumour type and associated genes with allelic loss for various tissue

Case No	Pathology	Cytology	Path-LOH*	Cyto-LOH†	Normal-LOH‡
1	Pancreatic cancer	Positive	k-ras, RIZ, VHL, PTEN, P53	k-ras, RIZ, VHL, PTEN, P53	No LOH
2	Pancreatic cancer	Positive	k-ras, RIZ, VHL, P16, P53	k-ras, RIZ, P16, P53	No LOH
3	Pancreatic cancer	Positive	RIZ, P16	RIZ, P16	No LOH
4	Pancreatic cancer	Positive	k-ras, VHL, APC, P16, PTEN	k-ras, VHL, APC, P16, PTEN	No LOH
5	Cholangiocarcinoma	Positive	VHL, APC, P16, P53	VHL, APC, P16, P53	No LOH
6	Cholangiocarcinoma	Positive	APC, PTEN	APC, PTEN	No LOH
7	Cholangiocarcinoma	Positive	VHL, PTEN, P53	VHL, PTEN, P53	No LOH
8	Cholangiocarcinoma	Positive	RIZ, APC, PTEN	RIZ, VHL, APC, PTEN	No LOH
9	Pancreatic cancer	Inconclusive	k-ras, VHL, APC, P16, P53	k-ras, VHL, APC, P16, P53	No LOH
10	Pancreatic cancer	Inconclusive	k-ras, VHL, APC	k-ras, VHL, APC	No LOH
11	Cholangiocarcinoma	Inconclusive	RIZ, APC	RIZ, APC	No LOH
12	Cholangiocarcinoma	Inconclusive	APC, P16, PTEN	VHL, APC, P16, PTEN	No LOH
13	Cholangiocarcinoma	Inconclusive	RIZ, VHL, P16, P53	VHL, P53	No LOH
14	Cholangiocarcinoma	Inconclusive	RIZ, APC, P16, PTEN	RIZ, APC, P16, PTEN	No LOH
15	Cholangiocarcinoma	Inconclusive	VHL, P16, P53	P16, P53	No LOH
16	Cholangiocarcinoma	Inconclusive	RIZ, APC, P16, PTEN	APC, P16, PTEN	No LOH
17	Cholangiocarcinoma	Inconclusive	RIZ, PTEN, P53	RIZ, PTEN	No LOH
18	Inflammatory	Inconclusive	No PATH	No LOH	No LOH
19	Inflammatory	Benign	No LOH	No LOH	No LOH
20	Inflammatory	Benign	No LOH	No LOH	No LOH
21	Inflammatory	Benign	No LOH	No LOH	No LOH
22	Inflammatory	Benign	No LOH	No LOH	No LOH
23	Inflammatory	Benign	No LOH	No LOH	No LOH
24	Inflammatory	Benign	No LOH	No LOH	No LOH
25	Inflammatory	Benign	No LOH	No LOH	No LOH
26	Inflammatory	Benign	No LOH	No LOH	No LOH

*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.

Table 3 Example of microdissection genotyping, manifesting perfect concordance of mutational change between cytological samples (by both the CA method and the WGA approach) and the surgical specimen

Gene	Non-neoplastic tissue	Positive cytology (CA)*	Positive cytology (WGA)	Positive cytology (WGA)	Histology tumour
RIZ	NI	NI	NI	NI	NI
RIZ	I	LOH T	LOH T	LOH T	LOH T
VHL	I	LOH B	LOH B	LOH B	LOH B
VHL	NI	NI	NI	NI	NI
APC	I	No LOH	No LOH	No LOH	No LOH
APC	I	No LOH	No LOH	No LOH	No LOH
P16	I	No LOH	No LOH	No LOH	No LOH
P16	I	No LOH	No LOH	No LOH	No LOH
PTEN	I	LOH B	LOH B	LOH B	LOH B
PTEN	I	LOH B	LOH B	LOH B	LOH B
KRAS	No MUT	12G-R	12G-R	12G-R	12G-R
P53	NI	NI	NI	NI	NI
P53	I	LOH T	LOH T	LOH T	LOH T

*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.

material. These difference were interpreted as minor mutational profile variations from intratumoral heterogeneity, given that the specimens were taken from different parts of the tumour within each case.⁴⁷⁻⁴⁸ Two examples of microdissection 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 concordant 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 determination 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 important drawbacks. The first is the requirement for relatively abundant cellular material as broad panel mutational genotyping 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 4 Example of microdissection genotyping, revealing a minor degree of discordance affecting two markers from six that exhibited mutational change

Gene	Non-neoplastic tissue	Positive cytology (CA)*	Positive cytology (WGA)	Positive cytology (WGA)	Histology tumour
RIZ	NI	NI	NI	NI	NI
RIZ	I	LOH T	LOH T	LOH T	LOH T
VHL	I	No LOH	LOH B	LOH B	LOH B
VHL	I	LOH T	No LOH	No LOH	LOH T
APC	NI	NI	NI	NI	NI
APC	NI	NI	NI	NI	NI
P16	I	No LOH	No LOH	No LOH	No LOH
P16	I	LOH T	LOH T	LOH T	LOH T
PTEN	NI	NI	NI	NI	NI
PTEN	NI	NI	NI	NI	NI
KRAS	No MUT	12G-D	12G-D	12G-D	12G-D
P53	NI	NI	NI	NI	NI
P53	I	LOH B	LOH B	LOH B	LOH B

*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 mutational 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 carry out 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 malignancy³¹), 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, dysplastic states, or benign disease. 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 accrual 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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