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Determination of sequential mutation accumulation in pancreas and bile duct brushing cytology

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Neoplastic progression is characterized by clonal expansion of tumor cells associated with accumulation of mutational damage. The timing of mutation acquisition could be of value in distinguishing preneoplastic conditions from early and advanced cancer as well as characterizing tumor aggressiveness and treatment response. Using quantitative methods applied to microdissected cell clusters selected according to cytomorphologic features, we sought to demonstrate the feasibility and efficacy for determining the time and course of mutation accumulation in pancreatobiliary cytology specimens. In all, 40 pancreatic duct and 21 biliary brushing cytology specimens were retrieved from the cytology database. Xylene-resistant markings were placed on the slide underside and coverslips removed. Clusters of benign, atypical and malignant cells were manually microdissected and DNA extracted. Mutations (allelic imbalance) (loss of heterozygosity) were quantitatively determined for a broad panel of 15 markers (1p, 3p, 5q, 9p, 10q, 17p, 17q, 21q, 22q) as well as point mutation in *K-ras-2* using PCR/capillary electrophoresis. Time course was based on earlier mutations having a higher proportion of mutant DNA for a particular marker. The descending frequency of detectable mutational involvement in pancreatic cytology was *K-ras-2* point mutation (58%), 3p25–26 and 17q21 (35%), 5q23 (33%), 1p36 (28%), followed by the remaining molecular markers. The descending frequency of mutational content in bile duct cytology was 17p13, 1p36, 3p25–26, and 5q23 followed by remaining molecular markers. *K-ras-2* point mutation was not seen in bile duct specimens. While there was overlap in the spectrum of mutational markers in pancreatic duct and biliary brushing cytology, the temporal profile was significantly different ($P<0.001$). Pancreatic and biliary neoplasia progression involves distinct subset of accumulated defined mutations. Determination of timing of the mutational damage in cytologic material could be incorporated in the work-up and help in making a more definitive diagnosis of malignancy in pancreatobiliary cytology specimens.
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Cytologic evaluation is increasingly being used as a first step in cancer detection, diagnosis and treatment planning.[1–3] However, pancreaticobiliary cytology can be challenging, since the differential diagnosis includes reactive states (pancreatic pseudocyst, pancreatitis, biliary stricture), low-grade neoplastic lesions (mucinous cystic neoplasms, intraductal papillary mucinous neoplasia) and malignant tumors (pancreatic adenocarcinoma, cholangiocarcinoma).[4–6] Although cytologic diagnosis can be definitive and specific, in over 50% of such specimens the diagnosis is indeterminate.[5,6] With an atypical but indeterminate diagnosis, surgery may be performed for nonmalignant disease based on clinical and imaging features. Alternatively, indeterminate cytology may lead to conservative measures with surveillance and repeat examinations for a malignant condition. Therefore, in some cases, an indeterminate diagnosis may contribute to miss early cancer when surgery would be effective and later only palliation may be feasible. Owing to occasional limitations of cytology, we investigated whether molecular methods can provide additional information that can reduce the rate of indeterminate diagnosis.

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The transformation from a benign to malignant phenotype is associated with sequential acquisition of mutational alterations.[7–9] Various tumor-suppressor genes are lost and oncogenes activated at different stages of tumor development. The time when each mutation occur may determine the differences in tumor biological behavior, can influence the rate of subsequent mutation acquisition, characterize phenotypic variability of the cancer type and help to distinguish preneoplastic conditions from cancer.[7–11] Therefore, it is reasonable to believe that two neoplasms with essentially the same mutational profile but acquired at a different time should behave in a different manner with respect to neoplastic features. In addition, molecular findings may assume greater importance as gene specific therapy is introduced and increasingly used for cancer treatment.

Cancer arises via a multistep process of mutational accumulation that is responsible for phenotypic expression of biological properties, such as tumor invasiveness, metastatic spread and treatment responsiveness.[7–9] Acquired mutations that confer significant growth advantage will induce clonal expansion of phenotypically more aggressive tumor cells.[12–14] This will result in increasing numbers of neoplastic cells bearing the mutation, which may be appreciated microscopically by increased cellular atypia. Mutational change that is not associated with positive growth advantage will be diluted as a genotypic alteration in the overall pool of proliferating tumor cells. It is important to distinguish between acquired mutations that induce clonal expansion *vs* acquired mutations occurring in the background and not associated with significant proliferative features.[15,16]

Mutations which are present in a large proportion of cells more likely have been acquired earlier then mutations present in lesser numbers of cells with more localized distribution.[10,11,17] These molecular changes have not yet been previously described in cytology specimens where such information could greatly impact patient management. Our objective was to extend molecular studies to cytology specimens to determine the feasibility and effectiveness of mutation temporal profile determination in the anticipation that such information can potentially be used to render a more definitive cytologic diagnosis for pancreatic and biliary neoplasms.

Materials and methods

A total of 40 pancreatic and 21 biliary duct brushing cytology specimens were retrieved from Allegheny General Hospital cytology archives for 1999–2004. Xylene-resistant markings were placed on the slide underside representing cellular targets for integrated molecular analysis. Clusters of normal ($n=10$), atypical ($n=20$) and malignant ($n=10$) appearing cells in papanicolaou-stained smears were manually microdissected under stereomicroscopic observation.[18,19] Mutational analysis was based on polymerase chain amplification reaction (PCR) for a broad panel of genomic markers associated with tumor suppressor gene loss and oncogene activation commonly involved in human pancreatic and biliary carcinogenesis.[20–22]

Aliquots (1 μl) of the microdissected cells were used in individual genotyping reactions for a panel of markers consisting of *K-ras-2* exon 1 for direct DNA sequencing and 15 allelic imbalance microsatellite markers situated at 1p34–36 (two markers), 3p25–26 (two markers), 5p23 (two markers), 9p21–23 (two markers), 10q23 (two markers), 17p13 (two markers), 17q21 (one marker), 21q23 (one marker) and 22q12 (one marker). The corresponding genes for the markers used are as follows: 1p markers: *CMM*; 3p markers: *VHL*; 5q markers: *APC*; 9p markers: *CDKN2A*; 10q markers: *PTENN*; 17p markers: *TP53*; 17q marker: *NME1*. PCR amplification was designed to generate amplicons measuring <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. The PCR products were analyzed by capillary electrophoresis on an ABI 3100 according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).[23] Allele peak heights and lengths were used to define the presence or absence of allelic imbalance (ie, loss of heterozygosity) for a given sample. Allelic imbalance was reported when the ratio of polymorphic allelic bands for a particular marker was beyond 95% confidence limits for the variation in peak heights for individual allele pairings derived from analysis using non-neoplastic specimen samples in preliminary studies. In general, this value was below 0.5 or above 2.0. DNA sequencing of *K-ras-2* exon 1 PCR amplified DNA was used to search for and characterize point mutations in codons 12 and 13. The peak heights of normal and mutant bases were used to determine the proportion of mutated and non-mutated cells in each microdissected sample. Replication was performed on multiple samples where sufficient numbers of cells were available and on individual markers to validate the genotyping results. The lower threshold for detection of significant allelic imbalance (LOH) was approximately 40% of microdissected cells whereas the threshold for point mutation detection was approximately 20%.

When the average value for normal sample allele peak height ratios is 1.00, allele ratios of 2.0 or 0.5 corresponded to 50% of the microdissected cells bearing allelic imbalance for that particular marker. The deviation from ideal normal ratio of 1.0 indicated which specific allele was affected. Based on a model of clonal expansion, the order of mutation acquisition could then be arranged in a sequence reflecting the proportion of cells affected

by specific microsatellite marker loss and point mutation. Markers displaying more extreme ratios are considered to have been acquired earlier. When two or more mutations, *K-ras-2* and/or allelic imbalance mutations, were detected, their time course of accumulation was determined by the proportion of total DNA manifesting the alteration. In the case of *K-ras-2* oncogene point mutation, involvement of 100% of cells was considered to be present when the density of the mutated base on sequencing autoradiography was equal to or greater than the normal sequence base pair.

Results

Cytology preparations generally provided a small but sufficient number of representative cells for molecular analysis. Individual clusters of target cells were microdissected and collected for broad panel genotyping. Every effort was made to replicate the analysis both at the microdissected target level and at the individual marker level when the specimen was limited.

The ratio of allelic peak heights is a quantitative measure of the proportion of mutated and nonmutated cells in the microdissected cytology sample (Figure 1). It should be noted that the nonmutated cells for a particular marker are composed of two groups: non-neoplastic cells included in the microdissection and neoplastic cells that do not bear that specific mutational change. The first is a fixed value for each microdissected sample and present in all mutational analyses for that sample. This normal cellular component enabled accurate determination of the status for each allelic imbalance marker and did not account for >5% of total microdissected cells.

The second component of nonmutated DNA associated with neoplastic cells reflects the timing of specific mutation acquisition in that mutational damage acquired early and causally linked to clonal expansion would be present in the greatest proportion of neoplastic cells (Figure 1). Since each mutational analysis, uses an equivalent amount of total sample DNA, significant differences in imbalance peak height ratios could be directly linked to the temporal pattern of specific marker mutation (Figure 1).

In the case of point mutational change, a similar analysis could be carried out (Figure 2). The ratio of peak heights of the normal and mutant bases correlated with the proportion of mutated and nonmutated cells (Figure 2).

The complete data for all patient specimens is shown in Table 1. In addition to discrimination of normal and mutated status, markers showing mutational change were separated according to specific allele affected by imbalance, specific form of *K-ras-2* point mutation and proportion of microdissected cells affected by mutational change. As all values are

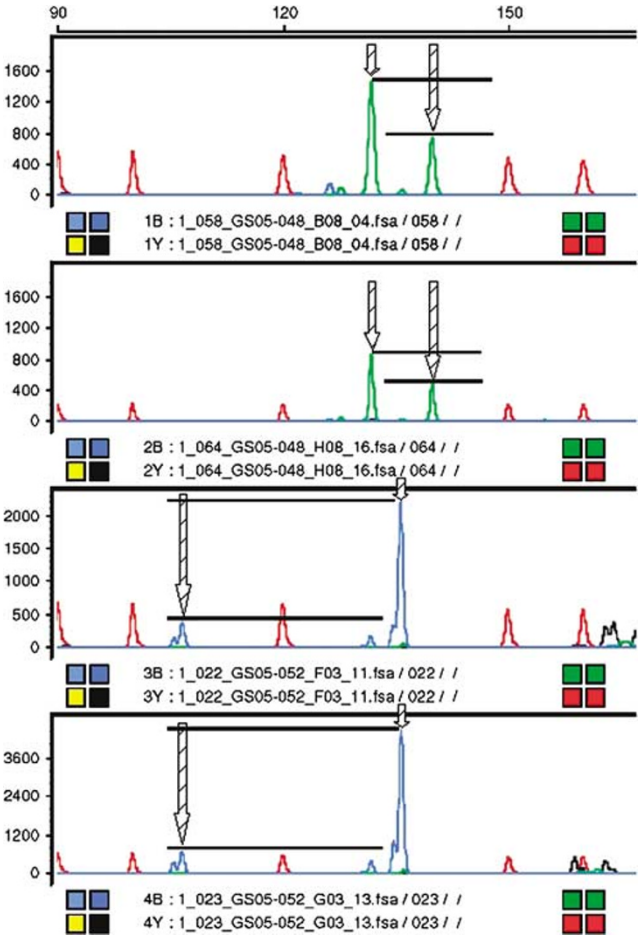

Figure 1 Using equivalent aliquots of microdissected sample DNA, certain markers show lower degrees of significant imbalance (LOH) (upper pair of samples) than other markers, which exhibit major imbalance (lower pair of samples). The quantitative degree of imbalance is indicated by arrows and horizontal lines reflecting difference in peak heights. This enables a determination of the temporal profile of mutation acquisition in which earlier mutations manifested more extreme degrees of imbalance.

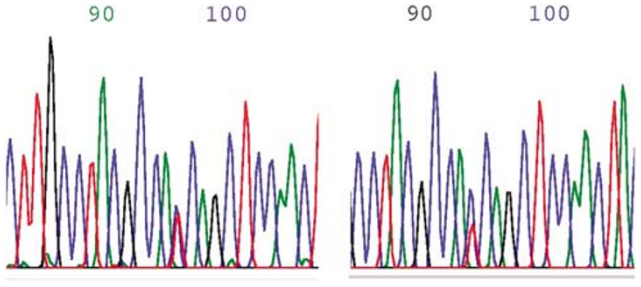

Figure 2 The same relationship between relative differences in peak height and temporal mutation acquisition apply to point mutation determination as for K-ras-2 exon 1. In the upper panel, the height of the normal band and the mutant band is nearly equivalent indicating that 100% of the microdissected cells are heterozygous for the oncogenic point mutation. In the lower panel, the mutant band is approximately 60% the height of the normal band indicating that 60% of the microdissected cells have acquired the point mutation.

Table 1 Complete data set for temporal analysis

	KRAS	1p36 D1S 1193	1p36 D1S 407	3p25 D3S 1539	3p25 D3S 2303	5q23 D5S 592	5q23 D5S 615	9p21 D9S 251	9p21 D9S 254	10q23 D10S 1173	10q23 D10S 520	17p13 D17S 1289	17p13 D17S 974	17q23 D17S 1161	21q23 D21S 144	22q12 D22S 532
Pancreas																
1	NO MUT	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	66%	NO LOH	NI	NO LOH	NO LOH	NO LOH
2	12-R	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH
2	12-R	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH
3	12-D	NO LOH	NI	NO LOH	NO LOH	51%	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH
3	12-D	NO LOH	NI	NO LOH	NO LOH	54%	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	58%
4	12-R	77%	NO LOH	NI	61%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	88%	NO LOH	NO LOH	NO LOH	NO LOH
4	12-R	71%	NO LOH	NI	59%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	88%	NO LOH	NO LOH	NO LOH	NO LOH
5	12-C	NI	91%	NO LOH	NO LOH	89%	86%	NO LOH	NI	NO LOH	NO LOH	NI	79%	76%	NI	NO LOH
5	12-C	NI	95%	NO LOH	NO LOH	93%	82%	NO LOH	NI	NO LOH	NO LOH	NI	77%	84%	NI	NO LOH
6	12-R	NO LOH	NO LOH	NO LOH	75%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
6	12-R	NO LOH	NO LOH	NO LOH	69%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
7	12-D	NI	NI	70%	NI	94%	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	83%	NO LOH	NO LOH	NO LOH
8	NO MUT	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	94%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH
9	12-R	97%	NI	88%	NO LOH	NI	80%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH
10	NO MUT	NO LOH	NO LOH	83%	NO LOH	NO LOH	NO LOH	NO LOH	60%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
10	NO MUT	NO LOH	NO LOH	67%	NO LOH	NO LOH	NO LOH	54%	52%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
11	12-D	NO LOH	NO LOH	60%	72%	NO LOH	NO LOH	NI	NO LOH	49%	81%	NO LOH	NO LOH	NO LOH	NO LOH	53%
11	12-D	NO LOH	NO LOH	56%	60%	NO LOH	NO LOH	NI	NO LOH	NO LOH	85%	NO LOH	NO LOH	NO LOH	NO LOH	51%
12	NO MUT	NO LOH	NO LOH	NI	98%	NO LOH	NO LOH	NI	NO LOH	NI	NI	NO LOH	NO LOH	70%	NO LOH	NO LOH
13	12-D	NI	58%	NO LOH	NO LOH	NO LOH	NO LOH	NI	98%	NO LOH	NO LOH	NO LOH	51%	59%	NO LOH	NI
14	NO MUT	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	51%	NO LOH	NI	NO LOH	67%	NO LOH	NI
15	12D	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	78%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	71%	NI	NI
15	12D	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	83%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	99%	NI	NI
16	12-V	NO LOH	NI	83%	NO LOH	NI	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	95%	NO LOH	NO LOH
16	12-V	NO LOH	NI	91%	73%	NI	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH	70%	62%	NO LOH	NO LOH
17	12-R	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
17	12-R	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
18	12-R	99%	NO LOH	NO LOH	91%	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	67%	NO LOH	NI
19	12-V	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH
20	12-R	NO LOH	NO LOH	81%	NO LOH	NI	92%	NO LOH	NO LOH	NI	95%	68%	73%	99%	NO LOH	NO LOH
21	NO MUT	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	54%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
22	12V	NI	58%	NO LOH	53%	NO LOH	62%	NI	NI	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH
22	12V	NI	62%	92%	NO LOH	NO LOH	71%	NI	NI	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH
23	NO MUT	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NI	90%	NI	NI
23	NO MUT	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NI	79%	NI	NI
24	NO MUT	NO LOH	90%	NO LOH	NI	NO LOH	NO LOH	89%	84%	NO LOH	NO LOH	61%	NI	NO LOH	50%	NO LOH
25	NO MUT	75%	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI
26	12-R	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH
27	NO MUT	NO LOH	NO LOH	NO LOH	NO LOH	56%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH
27	NO MUT	NO LOH	NO LOH	NO LOH	NO LOH	49%	59%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH
28	12-R	67%	82%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI
29	NO MUT	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	49%	NO LOH	NO LOH
30	NO MUT	NO LOH	NO LOH	NI	NO LOH	34%	43%	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI
30	NO MUT	NO LOH	NO LOH	NI	NO LOH	55%	39%	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI
31	NO MUT	NO LOH	NO LOH	NI	NO LOH	90%	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH
32	NO MUT	NO LOH	91%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH
32	NO MUT	NO LOH	58%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH
33	NO MUT	NI	NO LOH	NO LOH	NO LOH	60%	NO LOH	NO LOH	NO LOH	NO LOH	87%	NI	NO LOH	99%	NO LOH	NI
33	NO MUT	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	68%	NI	52%	NO LOH	NO LOH	NI
34	NO MUT	NO LOH	NI	NO LOH	NO LOH	61%	NI	NO LOH	NI	NI	NO LOH	NI	NO LOH	NI	NO LOH	NI
34	NO MUT	NO LOH	NI	NO LOH	NO LOH	62%	NI	NO LOH	NI	NI	NO LOH	NI	NO LOH	NI	NO LOH	NI
35	12D	48%	47%	NI	NO LOH	70%	84%	NI	NI	NO LOH	NI	NI	NI	NI	NI	NI
35	12D	NO LOH	NO LOH	NI	NO LOH	60%	53%	NI	NI	NO LOH	NI	NI	NI	NI	NI	NI
36	12-V	NO LOH	NO LOH	96%	89%	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH
37	NO MUT	NO LOH	NO LOH	NI	87%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH
38	12-D	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH
39	12-D	NO LOH	NI	NO LOH	NO LOH	NO LOH	58%	62%	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH
40	12D	NO LOH	NI	NI	61%	NO LOH	NO LOH	NI	56%	NO LOH	53%	NO LOH	NO LOH	NI	NO LOH	NO LOH
40	12D	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH	NI	51%	NO LOH	NO LOH	56%	NO LOH	NI	NO LOH	NO LOH

Table 1 Continued

	KRAS	1p36 D1S 1193	1p36 D1S 407	3p25 D3S 1539	3p25 D3S 2303	5q23 D5S 592	5q23 D5S 615	9p21 D9S 251	9p21 D9S 254	10q23 D10S 1173	10q23 D10S 520	17p13 D17S 1289	17p13 D17S 974	17q23 D17S 1161	21q23 D21S 144	22q12 D22S 532
Bile duct																
1	NO MUT	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH
2	NO MUT	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	71%	NO LOH	NI	NO LOH	NO LOH	NO LOH
2	NO MUT	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	73%	NO LOH	NI	NO LOH	NO LOH	NO LOH
3	NO MUT	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NI	NI
3	NO MUT	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NI	NI
4	NO MUT	55%	NO LOH	NI	NI	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI
4	NO MUT	49%	NO LOH	NI	NI	NO LOH	NI	NO LOH	52%	NI	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI
5	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
5	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH
6	NO MUT	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI
7	NO MUT	NO LOH	NI	NI	NO LOH	58%	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI
7	NO MUT	NO LOH	NI	NI	NO LOH	58%	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI
8	NO MUT	NI	NO LOH	NO LOH	NI	79%	68%	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH
8	NO MUT	NI	NO LOH	NO LOH	NI	69%	66%	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH
9	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH
10	NI	NO LOH	NO LOH	55%	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH
11	NO MUT	NO LOH	56%	NI	NO LOH	NI	NI	NI	NO LOH	NI	NI	58%	NO LOH	NO LOH	NI	NI
11	NO MUT	NO LOH	56%	NI	NO LOH	NI	NI	NI	NO LOH	NI	NI	77%	59%	NO LOH	NI	NI
12	NO MUT	NI	NO LOH	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI
13	NO MUT	NO LOH	NO LOH	NI	62%	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH
14	12-D	NI	60%	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NI	NI	57%	NI	NI	68%
14	NO MUT	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NI	NI	NO LOH	NI	NI	NO LOH
15	NO MUT	NO LOH	NO LOH	NO LOH	NI	63%	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	91%	NO LOH	NI	NO LOH
15	NO MUT	NO LOH	NO LOH	69%	NI	60%	NO LOH	NO LOH	NI	NO LOH	NI	NO LOH	91%	NO LOH	NI	NO LOH
16	NO MUT	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI
16	NO MUT	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	NI	NI	NO LOH	NI	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI
17	NO MUT	NI	NO LOH	NO LOH	NO LOH	60%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI
17	NO MUT	NI	NO LOH	NO LOH	NO LOH	60%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NI
18	12-V	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	99%	NI	NI	NO LOH
18	12-V	NO LOH	NI	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	NO LOH	97%	NI	NI	NO LOH
19	NO MUT	NO LOH	NI	NO LOH	51%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH
19	NO MUT	NO LOH	NI	NO LOH	63%	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH
20	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	73%	NI
20	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NI	NO LOH	NO LOH	NO LOH	NO LOH	NO LOH	71%	NI
21	NO MUT	53%	45%	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	68%	NI	46%	58%	NO LOH	NO LOH	NO LOH
21	NO MUT	84%	47%	NO LOH	NO LOH	NI	NO LOH	NO LOH	NI	99%	NI	67%	89%	NO LOH	NO LOH	NO LOH

derived from equivalent aliquots of the microdissected sample, markers exhibiting more extreme degrees of mutational content were acquired before markers showing lesser degree of mutational content. For each patient, the unique temporal profile of mutation acquisition could be determined with a representative example shown in Figure 3.

In the malignant cytology group, 4/10 patients had histology follow-up and carcinoma was confirmed in these cases. Out of 20 in the atypical cytology group, nine had histologic follow-up with carcinoma in 4/9 specimens. Out of 10 in the benign cytology cases, four had histology follow-up with 3/4 had a benign diagnosis.

Conclusions

The determination of the constellation of cancer related changes in a neoplastic specimen has received great interest due to genome wide techniques, such as DNA microarrays, comparative genomic hybridization and proteomic chip technology.[24–26] Timing of mutation acquisition may rightly be expected to exert profound effects on cancer biology and may also influence responsiveness to the treatment.[10,11]

Knowing that cancer evolves through a temporal process of mutation acquisition raises the question whether this sequence can be determined in clinical specimens. Mutations that are causally responsible for a significant increase in neoplastic behavior should be reflected within individual cells and across the tumor by the degree to which they induce clonal expansion of more aggressive tumor cells. We have shown previously that mutations present in virtually all clonal cells are likely to have been acquired early in tumorigenesis and may be largely responsible for phenotypic expression and cancer behavior.[27] In contrast, mutations that are focally

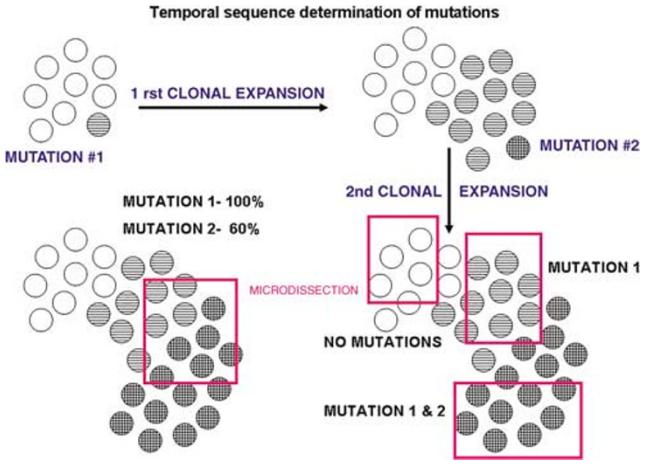

Figure 3 The temporal profile of sequential mutation acquisition can be represented by a schematic diagram highlighting the order to mutation accumulation. Mutations acquired early are present in multiple microdissection targets and at a higher proportion of affected cells. Mutations acquired later in temporal sequence tend to be more limited in topographic extent and at a relative lower percentage of affected cells. The temporal profile demonstrates the diversity that exists between different patients with the same histologic form of cancer.

distributed likely to have arrived later in cancer evolution and may be expected to contribute minimally to the overall phenotype of that neoplasm.[24]

We have shown that pancreatic and bile duct cancers share allelic imbalance damage for a subset of mutational markers (Table 1). The descending frequency of detectable mutational involvement in pancreatic cytology was *K-ras-2* point mutation (58%), 3p25–26 and 17q21 (35%), 5q23 (33%), 1p36 (28%), followed by the remaining molecular markers. *K-ras-2* point mutation occurred in all but 1 malignant case and was associated with abundant coexisting acquired mutational damage. The descending frequency of detectable mutational involvement in bile duct cytology was 17p13, 1p36, 3p25–26, 5q23 followed by remaining markers. While there was overlap in the spectrum of mutational markers, the temporal profile was significantly different ($P<0.001$). This supports the concept that tumorigenesis is not a random process, but one guided by factors that are as yet not fully understood.

In order to address the need for more definitive diagnosis in cytology, the correlation of molecular information appears to be a most productive option to undertake.[28–30] This information however must be presented in the context of microscopic findings that is essential to appropriately integrate molecular information. Mutational profiling is best carried out after microscopic evaluation of all available specimens to define the optimal targets for correlative molecular analysis. The microdissection platform, where small numbers of cells are obtained and then subdivided for broad panel analysis offers an effective means to generate significant amounts of data from limited size specimens. By utilizing quantitative means applied to multiple microdissection targets, the temporal profile can be developed generating patient specific information at the individual patient level.[31–35] In conclusion, we believed that the determination of sequential timing of mutational damage in cytologic material could be incorporated in the work-up and help to make more definitive diagnosis of malignancy in pancreatobiliary cytology specimens

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