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ARTICLE

Tiny but mighty: use of next generation sequencing on discarded cytocentrifuged bile duct brushing specimens to increase sensitivity of cytological diagnosis

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Abstract

Bile duct brushing (BDB) is used to evaluate pancreatobiliary lesions as it widely samples lesions with a low complication rate. Cytological evaluation of BDB is a specific but insensitive test. There is limited literature on the use of post-cytocentrifuged (PCC) samples, which are usually discarded, for next-generation sequencing (NGS) as an adjunct to cytological diagnosis of BDB. In this study we investigate whether molecular analysis by NGS of PCC specimens improves the sensitivity of diagnosis. PCC samples from 100 consecutive BDB specimens spanning 93 unique patients were retained. DNA was extracted and mutational analysis was performed agnostic of morphologic or clinical findings. Each BDB specimen was characterized as negative, atypical or positive based on morphological analysis by trained cytopathologists. Performance characteristics for mutational profiling and morphological analysis were calculated on the basis of clinicopathologic follow-up. There was sufficient clinicopathologic follow-up to classify 94 of 100 cases as either malignant ($n = 43$) or benign ($n = 51$). Based on morphologic analysis of cytology, these 94 cases were classified as either benign ($n = 55$), atypical ($n = 18$), or as at least suspicious or positive for malignancy ($n = 21$). Morphologic analysis of cytology showed a sensitivity of 49% and a specificity of 100% if atypical cases were considered negative. NGS revealed oncogenic alterations in 40/43 (93%) of malignant cases based on clinicopathologic follow-up. The most common alterations were in *KRAS* and *TP53*, observed in 77% and 49% of malignant cases respectively. No alterations were observed in the 51 benign cases classified based on clinicopathologic follow-up. Supplementing cytomorphologic analysis with molecular profiling of PCC by targeted NGS analysis increased the sensitivity to 93% and maintained specificity at 100%. This study provides evidence for the utility of NGS molecular profiling of PCC specimens to increase the sensitivity of BDB cytology samples, although studies with larger cohorts are needed to verify these findings.

Introduction

Pancreatobiliary malignancies, most commonly cholangiocarcinoma and pancreatic carcinoma, are associated with a dismal prognosis and limited treatment options. Cytologic brushings are a standard sampling technique for malignancy during endoscopic retrograde cholangiopancreatography (ERCP) as forceps biopsies are limited to certain anatomic locations within the pancreatobiliary tract. Timely identification of these malignancies is important for directing clinicians to management options such as stent placement, surveillance, or surgery [1, 2]. However, this is complicated by diagnostic challenges including differentiation of benign from malignant biliary strictures by imaging, sample failure of biopsy and brushing, and cytomorphologic identification of tumor cells with minimal morphologic malignant features [2].

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BDB cytology is very specific for malignant lesions, but its sensitivity remains low [3–5]. Adjunctive tests such as digital image analysis, assessment of *KRAS* mutation status, and fluorescence in situ hybridization (FISH) for polysomy have been used to improve on the sensitivity of cytology diagnosis [6–8]. The goal of this study is to evaluate the performance characteristics of molecular profiling of post-cytocentrifuged specimens (PCC) by targeted next-generation sequencing (NGS) analysis as an adjunctive test for high-risk neoplasia in BDB cytology specimens.

Materials and methods

Samples were obtained by standard ERCP at University Hospitals Cleveland Medical Center from patients with bile duct strictures from June 2018 to March 2019. A set of 100 bile duct brushing (BDB) samples was collected for cytological evaluation. The specimens were handled and processed according to the routine institutional workflow. All BDB were accepted, regardless of tumor content. The specimen tubes were received in CytoLyt® preservative solution (Hologic Inc., Marlborough, MA) with brush. The tubes were vortexed for 5 min to dislodge the cells from the brush. The brush was then removed and discarded. The specimen tube was then centrifuged at $1200 \times g$ for 5 min. The PCC was then poured into a tube. The pellet was then fixed in PreserveCyt for 20 min and used to prepare a ThinPrep slide. The PCC (~30 mL) was stored at 4 °C until DNA extraction (3–20 days) with no additional additives or fixatives. Supernatant specimens collected in CytoLyt and stored at 4 °C can reliably be tested by NGS for up to 75 days [9]. In the molecular laboratory, PCC specimens were centrifuged at $4500 \times g$ for 5 min and ~1 mL of the concentrated sample was collected and transferred to a 1.5-mL Eppendorf tube. This tube was then centrifuged at $10{,}000 \times g$ for 5 min to further concentrate the material for nucleic acid extraction. A cell pellet was not normally seen. The top fluid was removed and the remaining 50 to 100 µL of concentrated fluid was resuspended in 200 µL of phosphate-buffered saline for nucleic acid extraction with Maxwell RSC DNA and RNA kits (Promega Corporation, Madison, WI) according to the manufacturer's protocol for cytology samples. DNA was quantified using the Quantus Fluorometer (Promega Corporation) with the Maxwell RSC Instrument (Promega Corporation). Quantifiable nucleic acid was retrieved from all samples in the cohort. An input of 15 ng of genomic DNA was used for library preparation using the Solid Tumor Focus Assay as previously described [10]. For a list of genomic regions interrogated refer to Supplemental Table 2. The Ion Chef and HiQ View sequencing kits (Thermo Fisher Scientific, Waltham, MA) were used for template amplification and enrichment, and sequencing was performed on the Ion Torrent PGM (6–7 samples per v318 chip) or Ion GeneStudio S5 System (15–18 samples per 530 chip) sequencers (Thermo Fisher Scientific). Data generated was aligned to hg19 reference sequence. Variant calling was performed using the Torrent Variant Caller (v5.10) under somatic settings and a custom hotspot bed file that contained all COSMIC (v87) entries spanning the targeted amplicons. Gene amplifications were identified with Ion Reporter Software (v5.4; Thermo Fisher Scientific) using the Oncomine Focus workflow. Mutations of clinical significance were assigned by a board-certified molecular pathologist blinded to morphology and follow-up results.

A positive molecular result was defined by the presence of at least one pathologic variant and/or amplification in a known tumor-associated gene. In select molecular false-negative cases, assessment for the presence of mutations below the limit of detection (LoD) of the Solid Focus Tumor Assay (LoD variant allele frequency = 2%) was performed using the Oncomine Pan-Cancer Cell-Free Assay (LoD variant allele frequency = 0.1%; Thermo Fisher Scientific) per manufacturer protocol using the Ion Chef, Ion GeneStudio S5, and Ion Reporter Systems. The cytology diagnosis of each case was recorded. Medical records were reviewed to document patient demographics, clinical presentation, ERCP findings, concurrent endoscopic ultrasound (EUS) and EUS-fine needle aspiration (FNA), serum CA19-9 levels, serum lipase, serum CEA and pathological diagnoses of corresponding brushings as well as their clinical follow-up. Site of tumor origin of pancreatobiliary carcinoma was divided into "pancreatic" and "biliary" carcinomas based on clinical information. Cases reported as suspicious for malignancy by the cytopathologist were considered part of the positive cohort for this study. Based on the clinical practice in our institution of treating "atypical" cytology results similar to "negative" results, atypical cases were considered negative for the purposes of calculating sensitivity and specificity of cytomorphologic evaluation for malignancy. Sensitivity and specificity were calculated using standard 2×2 contingency tables for cases with confirmed diagnostic pathology.

Results

The mean age of patients in this cohort was 66 years, (range, 23–98 years) and included 46 men and 47 women. There were 51 benign cases and 43 malignant cases: 34 pancreatic ductal adenocarcinoma (PDA) and 9 biliary carcinomas (8 cholangiocarcinoma and 1 gall bladder carcinoma). In addition, there were 6 indeterminate cases with insufficient clinicopathologic follow-up. Cytology results were negative in 55 patients, atypical in 18 cases, and at

least suspicious or positive for malignancy in 21 cases (Table 1). Using these cohorts, the sensitivity and specificity of cytomorphological evaluation were 49% and 100%, respectively, based on clinical follow-up results.

Serum markers were available in a subset of cases: CA19-9 in 47 cases, lipase in 45 cases, and CEA in 46 cases. Utilizing the cutoff values at our institution, the sensitivities and specificities of elevated serum markers for the detection of malignancy were 54% and 80% for CA19-9, 47% and 100% for lipase, and 28% and 80% for CEA, respectively (Table 3).

Quantifiable nucleic acid was retrieved from all samples in the cohort. Fifty-four cases were negative by NGS and 40 cases were positive. NGS revealed genomic alterations of clinical significance in 40/43 malignant cases (93%) (Fig. 1). Five of the 55 cases classified as benign by cytology were positive by molecular (NGS) analysis of the PCC sample, and the patients from all five of the cases had malignant disease on clinical follow-up (Table 1 and Fig. 1). Fourteen of the 18 cases classified as atypical by cytology were positive by molecular analysis, and the patients from all 14 of these cases had malignant disease on clinical follow-up (Table 1 and Fig. 1). All 21 cases diagnosed as adenocarcinoma or suspicious by cytology were positive by molecular analysis and were malignant on clinical follow-up (Table 1 and Fig. 1). The sensitivity and specificity of NGS in cases with adenocarcinoma/suspicious cytomorphologic diagnosis was 100%. The most commonly mutated gene identified by NGS was *KRAS*, with mutations present in 30 of the 39 unique patient malignant cases, followed by *TP53* which was observed in 19 of 30 cases (Table 2). Additional alterations were found in *CDKN2A* (6 of 39), *PIK3CA* (2 of 39), *SMAD4* (3 of 39), and *ERBB2* (2 of 39), see Fig. 1. Mutations were also detected in *B2M,*

Table 1 Correlation of cytology and molecular findings in cases with adequate clinical follow-up.

Cytological diagnosis	Molecular (NGS)	Clinical follow-up		Total
		Benign	Malignant	
Benign	Negative	48	2	50
($n = 55$)	Positive	0	5	5
Atypical	Negative	3	1	4
($n = 18$)	Positive	0	14	14
Suspicious or positive for carcinoma	Negative	0	0	0
($n = 21$)	Positive	0	21	21
Total		51	43	94

Table 2 Genomic alterations identified in pancreatic adenocarcinomas in this study as compared with previous reported studies.

	This study ($n = 39$)	Bailey et al.[21] ($n = 456$)	Biankin et al.[27] ($n = 99$)	Witkiewicz et al.[26] ($n = 105$)
KRAS	76.9	89.8	94.9	91.7
TP53	48.7	66.1	33.3	50.5
SMAD4	7.6	22.5	16.2	19.3
CDKN2A	15.3	18.5	2.0	5.5
PIK3CA	5.1	1.6	0.0	3.7
U2AF1	2.6	1.6	0.0	2.8
ERBB2	5.1	0.8	0.0	0.9
FBXW7	2.6	0.5	2.0	0.9
PTEN	2.6	0.3	0.0	0.9

Values shown represent percentage in each data set.

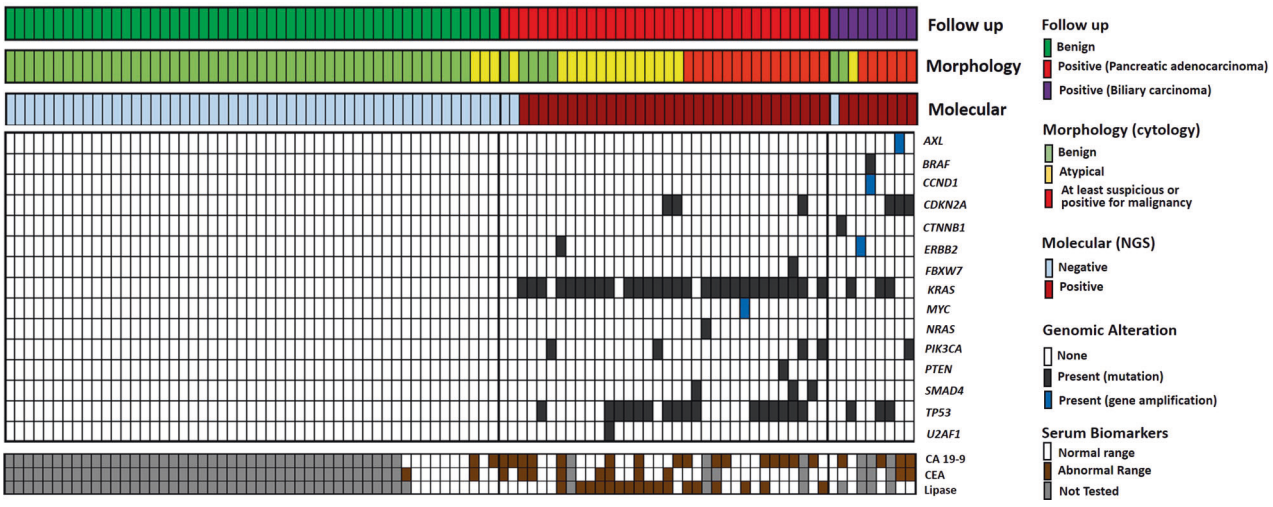

Fig. 1 Correlation of morphocytological diagnosis, detected genomic alterations, and serum markers with clinical diagnosis on patient follow-up. For serum markers, the following levels were used to define abnormal: CA19-9 > 30 U/ml, lipase > 82 U/L, and CEA > 2.5 ng/ml.

BRAF, CCND1, CTNNB1, FBXW7, NF1, *PTEN* and *U2AF1* (Fig. 1, Supplemental Table 1). Twenty-seven of 43 malignant cases showed two or more gene alterations (Fig. 1).

Of the four cases classified as atypical by cytology and negative by molecular analysis, one was malignant and three were diagnosed as pancreatitis on further clinical follow-up. Overall, compared with clinical follow-up data, NGS testing resulted in three-false-negative results compared with 22-false-negative results by cytomorphology alone. The three-false-negative samples by molecular analysis of PCC were reflexed to a more sensitive assay (Oncomine Pan-Cancer Cell-Free Assay) which was able to detect low-level (variant allele frequency below 1%) pathogenic mutations in two of the three cases: *KRAS* p.G12D and *PIK3CA* p.G1049R in one case and *KRAS* p.G12V and *ERBB2* p.S310F in the other case. The remaining false-negative sample by molecular analysis, which was also negative by cytology, did not show any detectable mutations with the more sensitive assay and was diagnosed as a cholangiocarcinoma on clinical follow-up. Retrospective review of the cytomorphology of this case did not show any atypical or malignant clusters and suggested that the sample was not representative of the lesion.

Discussion

BDB are often used as the initial investigative pathology test for pancreatobiliary tract lesions as they widely sample the bile duct and have a low complication rate. Though the specificity of BDB cytology approaches 100%, the sensitivity is limited [11–14]. A meta-analysis of BDB cytology reported a sensitivity and specificity of 45% and 99%, respectively [15]. In concordance with previous reports, the present study reveals a sensitivity of 49% and a specificity of 100% by morphological analysis of cytology specimens in a highly specialized pathology department at a tertiary care center. Elevated serum markers did not fare much better in our cohort, with the sensitivities of CA-19-9, lipase and CEA reaching only 54%, 47% and 28%, respectively (Table 3). When molecular analysis by targeted NGS assay on PCC specimens was added to the diagnostic workflow, the sensitivity improved to 93% (Table 3), which is far greater than the previously reported sensitivity for BDB [11–14]. We acknowledge that this study needs to be verified with a larger set and with samples from other institutions.

Studies have reported a wide variety of reasons for the low sensitivity of BDB cytology including low cellular yield, sampling difficulties, and interpretation errors. Interpretation can be particularly problematic in limited-cellularity specimens that are often obscured by frequent ulceration, inflammation, and stent-related atypia. In addition, well-differentiated adenocarcinomas and the presence of confounding factors such as primary sclerosing cholangitis can make the distinction between reactive biliary epithelium and neoplasia particularly challenging [14, 16]. Therefore, the diagnosis of biliary strictures as benign or malignant requires a multidisciplinary approach including clinical, imaging, and pathologic findings.

Adjunctive tests such as digital image analysis, assessment of *KRAS* mutation status, and FISH for polysomy have been used to improve on the sensitivity of cytology [6–8, 17–20]. The sensitivity of digital image analysis varies from 14% to 48% and mildly increases the sensitivity when combined with FISH [6, 7]. In one study, quantitative PCR alone for *KRAS* mutations had a sensitivity of 47% for the detection of malignancy, which was likely limited by the restriction of the analysis to a single gene [8, 21]. Multicolor FISH using the UroVysion probe set (Abbott Molecular Inc., Des Plaines, IL) demonstrates sensitivities for the detection of malignancy that range from 35 to 60% [6–8, 22]. However, this technique is labor intensive, costly, requires microscopy skills, and can be difficult to interpret due to nuclear overlap.

Mutation profiling has been attempted using traditional single-gene analysis, which is commonly performed with Sanger sequencing [23]. However this is relatively low in sensitivity and time- and labor-intensive. Furthermore,

Table 3 Sensitivities and specificities of serum markers, cytology evaluation, and molecular evaluation.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Elevated CA-19-9 ($n = 47$)	0.54 [0.37–0.70]	0.80 [0.44–0.96]	0.91 [0.69–0.98]	0.32 [0.16–0.54]
Elevated lipase ($n = 45$)	0.47 [0.31–0.64]	1.00 [0.63–1.00]	1.00 [0.77–1.00]	0.32 [0.17–0.52]
Elevated CEA ($n = 46$)	0.28 [0.15–0.45]	0.80 [0.44–0.96]	0.83 [0.51–0.97]	0.24 [0.11–0.42]
Pathologic evaluation (cytology) ($n = 94$)	0.49 [0.34–0.64]	1.00 [0.91–1.00]	1.00 [0.81–1.00]	0.70 [0.58–0.80]
Molecular evaluation (NGS) ($n = 94$)	0.93 [0.80–0.98]	1.00 [0.91–1.00]	1.00 [0.89–1.00]	0.94 [0.84–0.99]

CI confidence interval, *PPV* positive predictive value, *NPV* negative predictive value.

Elevated was defined as: CA19-9 > 30 U/ml, lipas > 82 U/L, and CEA > 2.5 ng/ml.

substantial amounts of DNA are required; thus the simultaneous evaluation of several genes within a small specimen is not possible. NGS circumvents this problem as it is able to detect numerous alterations simultaneously using limited amounts of DNA.

The importance of an accurate and timely diagnosis cannot be overstated in the case of pancreatobiliary lesions. Not only does prompt treatment of early stage malignancy offer a better prognosis, but it is also important to diagnose patients with biliary lesions accurately since 15–24% of patients who undergo surgery for suspicious biliary lesions are found to have benign pathology on resection [24, 25]. A quick and accurate diagnosis ensures that patients with malignancy are given the best chance for recovery, and patients with benign disease are spared from unnecessary and complicated surgery with a high rate of morbidity.

When comparing NGS testing to clinicopathologic follow-up, our analysis found no false positive results among 51 benign samples. *KRAS* mutations were the most commonly identified alterations. It is important to note that in 10 malignant cases, defined based on cytological and/or clinical findings, *KRAS* mutations were the only alterations identified. As a single genomic finding cannot define malignancy, molecular data should be used in the context of morphologic findings, clinical suspicion, and other laboratory findings. Our results are in concordance with larger molecular-integrated characterizations of pancreatic adenocarcinomas, and identified other potentially targetable alterations in *PIK3CA, ERBB2*, *BRAF*, and *PTEN* (Supplemental Table 1) [26–28]. The rates of *SMAD4* and *PIK3CA* alterations are significantly different in the present study compared with those described by others (Table 3). One possible explanation is that this study is much smaller in size and thus some differences in less commonly mutated genes like *PIK3CA* may be observed as sampling differences. As for *SMAD4*, only specific hotspots in exon 9 and 12 are covered in our analysis and thus a smaller number of mutations would be expected than in investigations including coverage of the entire gene. NGS testing alone produced three false-negative results. Using a more sensitive NGS test, mutations were detected in two of these three false-negative cases. Possible explanations for the false-negative result include minimal involvement of the biliary system, low level of tumor DNA in the sample, or the limitations of the assays to only assess mutations in a subset of commonly altered genes. The sensitivity of NGS for detecting malignancy could potentially be improved with an expanded gene panel and more sensitive target enrichment methods.

Microscopic examination of BDB samples is among the most challenging areas of cytology practice. Depending upon the precise location of biopsy or cytobrush sampling, sites representing the most advanced disease progression may be missed, thereby reducing the sensitivity for advanced dysplasia or cancer. Molecular profiling by targeted NGS to detect cancer-associated changes could significantly improve the detection of malignancy [23, 29]. There are challenges in the clinical application of NGS, including defining the best and most cost-effective markers to survey, integrating molecular workflows, and optimizing specimen handling to best preserve and complement existing cytology practice [28]. NGS is useful in detecting smaller quantities of mutant DNA in a larger background of normal DNA as well as evaluating numerous genes or targets simultaneously [30]. It has been proven to be useful in detecting cancer DNA alterations in a variety of cytology specimens such as Papanicolaou test fluid, bladder washings, stool samples, pancreatic cyst fluid, blood, and post-centrifugation specimens [9, 31–37]. This study illustrates the clinical utility of targeted NGS in the evaluation of BDBs for malignancy. We demonstrate that NGS is a useful adjunct assay and increases the sensitivity of cytology for the detection of malignancy. Our study is unique, as we demonstrate the viability of using PCC specimens for NGS testing. Here, we were able to successfully identify mutations in this specimen type, which is otherwise discarded, therefore the collection of the specimen for NGS testing did not compromise cytology evaluation nor did it require additional material to be taken from the patients.

Although in this study we investigated the utility of molecular profiling with every pancreatobiliary stricture cytology sample, this is not the only viable approach in clinical practice. Since cytology is highly specific, some institutions may opt to perform molecular profiling only in cases with negative cytology or in cases with atypical cytology or high clinical suspicion for malignancy [29]. In our study, we found that the sensitivity of NGS in cases with atypical cytomorphologic diagnosis was 93% and specificity was 100%. The sensitivity and specificity of NGS in cytology with adenocarcinoma/suspicious diagnosis was both 100%. Therefore, these integrated diagnostic approaches may increase the information available for preoperative clinical decision-making, especially in cases with atypical cytology results.

Here, we demonstrate that NGS testing of PCG specimens can be used as an adjunct to cytologic evaluation for both increasing the sensitivity of pathologic workup as well as identifying potential targetable alterations in limited but valuable cytology specimens. Further prospective studies are needed to distinguish the merits of different approaches of incorporating molecular profiling of PCC specimens in the management of patients with pancreatobiliary strictures, as well as to clarify the performance characteristics of targeted NGS analysis as an ancillary test for cytology.

Author contributions AH, NJ, HNG, CWM, PB, JW: Identified samples, rendered cytopathology diagnosis, and revised the manuscript. AH, CWM, NS: Conceived and designed the study. AH, NS: Analyzed the data. AH, JY, NS: Wrote the manuscript and created figures.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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