Review Article

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Cytology of Bile Duct Brushings: Streaming Ahead with Time

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ABSTRACT

Endoscopic retrograde brush cytology of the biliary duct is an established tool for evaluation of obstructive biliary strictures or screening of primary sclerosing cholangitis (PSC) patients for dysplasia. It is a simple, minimally invasive procedure that can be performed during a therapeutic ERCP. Most authors have reported a sensitivity of 30-60% and a specificity of 90-100%. A positive result can be a reliable indicator of malignant neoplasm. However, there is no standardized reporting terminology designed specifically for bile duct brushings. Majority of bile duct brushings yield either benign ductal epithelium, reactive atypia of ductal epithelium or suspicious/positive for malignancy. Diagnosing malignancy in bile duct brushings is based on a constellation of cytologic features, and consideration of the overall picture- clinical presentation, radiology/endoscopy findings, etc. Different studies have highlighted various key features for diagnosing malignancy in bile duct brushings. Features most consistently associated with a malignant category are- loss of honeycomb architecture, 3D clusters, high n:c ratio, anisonucleosis (≥1:4 variation), irregular nuclear outlines, coarse clumped chromatin, and single malignant cells in the background. The utility of biliary brush cytology has been expanded by using FISH, immunocytochemistry, and Next-generation sequencing.

Keywords: *Endoscopic, Biliary brush, Cytology, Dysplasia, Bile duct*

Introduction

Introduction of flexible fiberoptic endoscopy (by Hirchowitz and Curtiss in 1950s) brought a paradigm shift in the field of gastroenterology as well as gastrointestinal pathology, by enabling a plethora of diagnostic and therapeutic procedures, as well as collection of tissue biopsy, aspiration cytology and brush cytology. In 1968, a group of physicians from the George Washington University Medical Center initiated the clinical application of Endoscopic Retrograde Cholangiopancreatography (ERCP). Use of brush cytology with ERCP of the biliary and pancreatic ducts was introduced by Osnes et al in 1975 [1]. Since then, brush cytology of the biliary tract has become an established tool for evaluation of obstructive biliary strictures or screening of patients for dysplasia. The main objective of obtaining cytology from pancreatobiliary tract is to detect a dysplastic process before it evolves into an invasive carcinoma, and early initiation of palliative care if the tumor is unresectable.

In 2015, the Papanicolaou Society of Cytopathology published standardized reporting terminology for the sign out of pancreatobiliary cytology. This system was composed of six categories, each with well-defined criteria, estimated risks for malignancy, and management recommendations [2]. However, no cytologic criteria were defined specifically for bile duct brushings in this reporting system (Table 1). Also, malignancy risks for the PSCSRPC (Papanicolaou Society of Cytopathology System for Reporting Pancreaticobiliary Cytology) categories for bile duct brushing specimens are widely variable in the available literature. Moreover, many studies in biliary brush cytology have not used the criteria and/or categories of the PSCRPC [3].

Discussion

There are several techniques for acquiring bile duct cytology, namely Percutaneous transhepatic aspiration (PTHA), ERCP-guided bile duct aspiration (BDA), ERCP guided bile duct brushing (BDB) and Endoscopic Ultrasound guided bile duct fine needle aspiration.

ERCP guided bile duct aspiration

This technique involves the use of a biliary catheter for aspiration of luminal bile duct contents (exfoliated cells, bile and any extraneous contents, if present). It is a simple method with a disappointingly low sensitivity (6-32%) for detecting biliary malignancy, and therefore, has been superseded by bile duct brushing cytology [4,5]. However, this technique can be applied to specimens collected through a chronic biliary drainage catheter [6].

Table 1: Salient features of different bile duct brushing categories.

CATEGORY	Salient features
Non-diagnostic	Either acellular specimen or substantial artifactual changes or contaminant gastrointestinal epithelium only.
Negative	Cohesive sheets of cells arranged in a flat honeycomb pattern, with retained cell polarity, smooth nuclear outlines and pale, homogeneous chromatin.
Atypical	Cell clusters with mild nuclear overlapping & disorganization, enlarged nuclei with low n:c ratios, overall nuclear homogeneity and occasionally prominent nucleoli. Inflammatory cells can be associated.
Suspicious for Malignancy	Degree of atypia is qualitatively and/or quantitatively insufficient for a conclusive diagnosis of malignancy- usually the malignant cells are too few, or the context (age/ symptoms/endoscopy/radiology) is missing.
Malignant	Mostly increased cellularity is present, along with nuclear crowding (loss of honeycomb architecture, 3D clusters, nuclear overlap), high n:c ratio, irregular nuclear membranes, anisonucleosis (≥1:4 variation), coarse clumped chromatin Presence of a two-cell populations and single malignant cells is important.

Table 2: Sensitivity and Specificity of bile duct brushing cytology in various studies.

	Sensitivity	Specificity
Ferrari Jr (1994)	56.2%	100%
Cohen (1995)	83%	98%
Lee (1995)	37%	100%
Kocjan (1998)	44%	100%
Renshaw (1998)	36%	95%
Stewart (2001)	59.8%	98.1%
Govil (2002)	68%	100%
Mahmoudi (2008)	61%	98%
Stoos-Veić (2010)	71%	100%
Selvaggi (2016)	38%	95%
Mehmood (2016)	65.3%	100%
Avadhani (2017)	69%	85%
Layfield (2022)	64%	74%

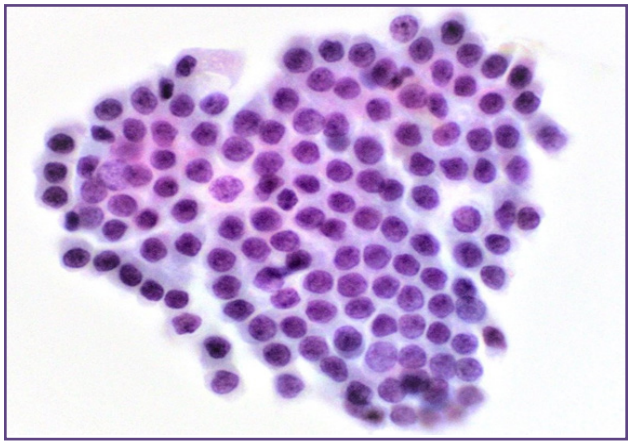

Fig. 1: Sheet of benign bile duct epithelium. Note the flat, honeycomb architecture with cellular equidistance and uniformity, rounded nuclei with smooth outlines and pale, homogeneous chromatin (Papanicolaou stain × 400).

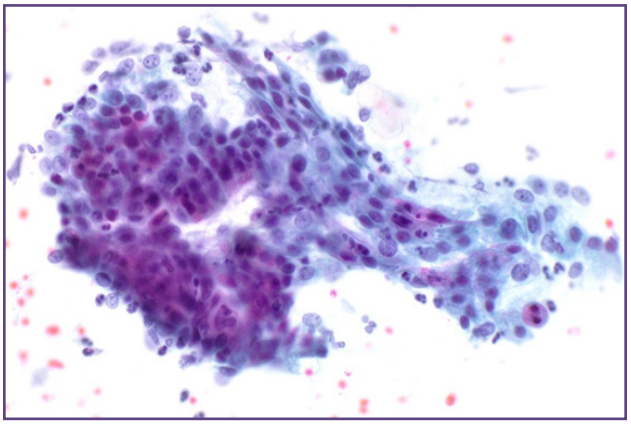

Fig. 2: Stent related reactive atypia: Sheet of ductal cells with dense cytoplasm, cytoplasmic stretching, mild anisocytosis & nuclear overlap, and prominent nucleoli. Note the interspersed neutrophils interspersed among the ductal cells (Papanicolaou stain × 400).

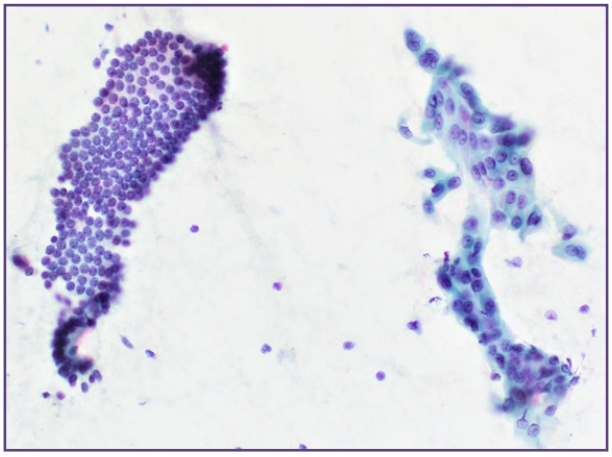

Fig. 3: Normal vs Reactive: Two-cell population showing a flat honeycomb sheet of evenly distributed benign ductal cells next to a cluster of ductal cells exhibiting reactive atypia (slight nucleomegaly with nuclear overlap, loss of polarity, low n:c ratio, stretched-out shape, prominent nucleoli) (Papanicolaou stain × 400).

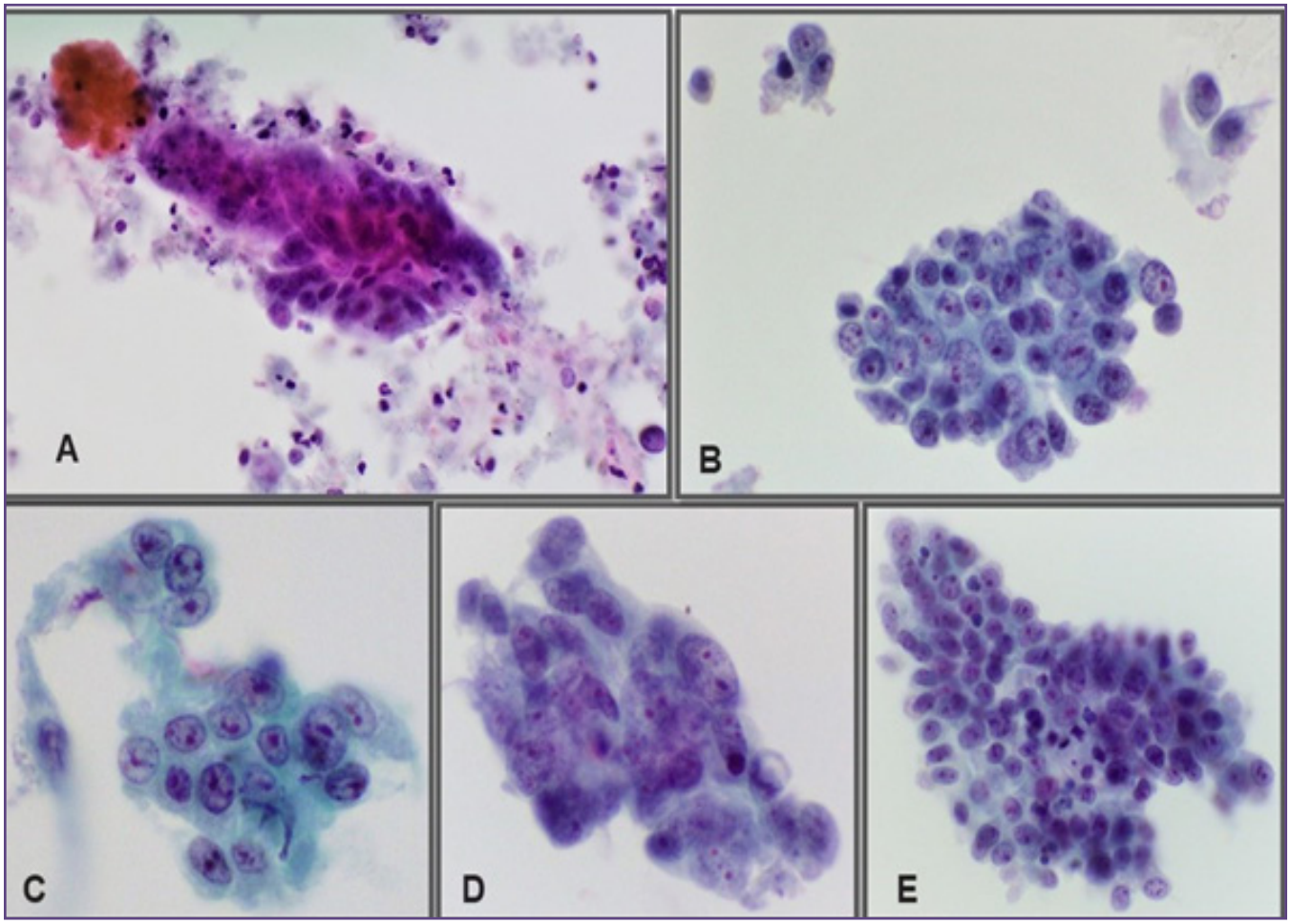

Fig. 4: Different faces of malignant ductal cells: All clusters have marked nuclear overlapping, irregular nuclear outlines, high n:c ratio, coarse chromatin and significant anisocytosis. Special features to be noted in each of these images are- Tumor diathesis (A), Hypochromatic nuclei (B), Single cells in background (C), 3D cluster (D), Hyperchromatic, coarse nuclei (E) (Papanicolaou stain× 400).

ERCP guided Bile duct brushing (BDB) or Endobiliary brush cytology

This is the traditional method for collecting tissue from the bile duct in the setting of a stricture [7]. The technique involves use of an ERCP guided cytology brush (Cytobrush) which is guided over a wire and passed into the biliary tract where it scrapes against the biliary mucosa and exfoliates superficial mucosa cells which then get trapped in its bristles. Repeated back and forth movements of the brush across a biliary stricture results in greater exfoliation, and better sampling. After sample collection, the brush is retracted back into its sheath and entire device is removed from the endoscope. The standard brush is 1.5 cm long with bristles oriented at 90° on a 6F sheath [6]. A newer 5cm long brush was introduced which has 3mm diameter, stiffer bristles oriented at 45° on a 7F sheath. But the sample quality between the two brushes was found to be similar (27% vs 30%) [8]. After the procedure, the material retrieved from the brush is smeared onto glass slides. Remaining material is washed into a fixative solution. The brush is agitated to dislodge any additional cells into the fixative. Any residual material may be employed for cell block preparation following fixation in formalin [6]. Alternatively, the brush may be cut off from the catheter and placed in a fixative solution. In the laboratory, the sample is agitated, and usually a ThinPrep slide is prepared.

There are several advantages of BDB- it is minimally invasive, has a rapid turnaround time, widely samples the biliary tract, and can be performed during a therapeutic ERCP procedure. Brush smears are usually rich in cells, and, if the preparation is fixed immediately, cell preservation is excellent [9].It has a low complication rate and a very high diagnostic specificity. A positive result can be a reliable indicator of malignant neoplasm, thus obviating the need for additional invasive diagnostic procedures. BDB has special role in the surveillance of patients with primary sclerosing cholangitis which is associated with a high risk of developing cholangiocarcinoma. Early detection of malignancy in these patients may improve survival by enabling a liver transplantation.

Diagnostic accuracy has varied but most authors have reported a sensitivity of between 30 and 60% and a specificity ranging from 90 to 100% for diagnosing maligancy (Table 2) [9-22]. Like any technique, BDB has its faults, chiefly being its low sensitivity rate. Negative results do not rule out a malignant process. The reported low sensitivity may be attributable to the inadequate sampling of submucosal tumors, difficult sampling (in cases of extrinsic compression of biliary tract by extramural tumors or extensive desmoplasia), significant artifacts from tissue crushing and distortion, interpretation errors and suboptimal slide preparation [23]. These are some of the most challenging cytologic specimens to evaluate due in part to poor specimen quality and quantity, as well as frequent ulceration, inflammation, and stent-related marked reactive atypia of the biliary epithelium that may make the distinction of benign or reactive biliary epithelium from neoplasms particularly challenging and result in a false-positive diagnosis of malignancy [24]. Therefore, *the threshold for malignant diagnosis is generally high in BDB cytology* [10]. A significant number of false-negative results may be due to the high frequency of deceptively benign-appearing carcinomas in this site e.g. well-differentiated mucinous or papillary tumors [25].

Several methods have been proposed to improve the diagnostic yield in BDB cytology- these include repeated sampling, stricture dilatation, use of ancillary techniques like Fluorescence in-situ hybridization (FISH) and flow cytometry, and most importantly, having an experienced endoscopist as well as a cytopathologist with prior experience in biliary brushing cytology. Studies have shown that higher frequency of positive yield has been associated with older age, stricture length of >1 cm, CA 19.9 level of >300 and proximal CBD strictures [18,26].

Non-diagnostic

The inability to make a diagnosis or meaningful interpretation from a bile duct brushing specimen by a pathologist relative to the lesion sampled indicates that cytology is correlated with imaging and ancillary testing, and deemed non-diagnostic. Non-diagnostic categorization may be due to- acellular specimen, specimen with substantial artifactual changes precluding evaluation of the specimen, contaminant gastrointestinal epithelium only, etc. Any cellular atypia precludes a nondiagnostic report [2,3].

Negative for malignancy:

Bile duct brushings may be negative in cases of sampling of either benign or reactive bile duct epithelium. **Benign bile duct brushings:** These are mainly composed of cohesive sheets of cells arranged in a flat honeycomb pattern with round to oval nuclei, retained cell polarity (i.e. basally located nuclei), low nuclear:cytoplasmic (n:c) ratios, smooth nuclear outlines and homogeneous pale chromatin. Small, indistinct nucleoli may be seen but necrosis or mitoses are never seen [12,13].

Bile duct epithelium in reactive conditions: Reactive atypia of bile duct epithelium may be seen mainly due to biliary stones, stents, infections, autoimmune cholangitis, primary sclerosing cholangitis, cholangiopathies (related to ischemia, radiotherapy), iatrogenic injury to bile ducts secondary to cholecystectomy, liver transplant, biliary-

enteric anastomoses, portocaval shunt, etc. Reactive biliary epithelium can demonstrate very pronounced atypia which can be sometimes difficult to differentiate from neoplastic conditions. These changes can be present in *varying degrees of gradation, resulting in a spectrum of cell populations* [10]. The epithelial cells usually retain their flat sheet architecture but appear mildly disorganized or crowded. Mild nuclear enlargement (upto 2X) and mild anisonucleosis may be seen; however, n:c ratio is usually low. Nuclear outlines maybe smooth or mildly irregular. The chromatin is mostly fine, but nucleoli may be prominent. Prominent nucleoli can be seen in marked reactive atypia in the context of stones, stents, primary sclerosing cholangitis, etc. The background may show acute inflammation and even necrosis. Inflammatory cells are commonly infiltrating between the ductal cells [12-13]. Low n:c ratios, the presence of inflammatory cells and gradation of cellular changes are helpful features to prevent overcall in atypical reactive cases [10]. Even though BDB in cases of PSC can show significant reactive atypia, one should on the look-out for features of dysplasia since PSC is a pre-malignant condition.

Atypical:

Smears categorized as 'Atypical' show nuclear, cytoplasmic, or architectural features that are not consistent with normal or reactive cellular changes but are insufficient for being diagnosed as suspicious or positive for a neoplastic process. Follow-up evaluation is warranted; follow-up histology in this category usually shows reactive atypia and low grade dysplastic (BilIN 1) lesions, although a significant proportion may have malignant outcomes. Since 'Atypia' is an indeterminate category, it should be used sparingly [10]. Features of an 'atypical' bile duct brushing are- flat, cohesive monolayered sheets of epithelial cells with mild nuclear overlapping and enlarged, slightly irregular nuclei with low n:c ratio. The nuclei may show mild enlargement with overlapping and slightly irregular outlines, but n:c ratio is mostly retained [12]. Stratification of the 'atypical' category into 'atypical favor reactive,' 'atypical, not otherwise specified' and 'atypical, suspicious for malignancy' improves diagnostic accuracy. The 'atypical suspicious for malignancy' category has a follow-up similar to the 'malignant' category while the 'atypical favor reactive' category is associated with a clinical outcome similar to that of the "benign" category [11].

Suspicious for malignancy:

This category includes specimens featuring greater dysplasia than seen in *Atypical* category, but the features are qualitatively and/or quantitatively insufficient for a conclusive diagnosis of malignancy. Histologic follow-up of the lesions assigned to this category demonstrate high-grade dysplasia as well as carcinomas of the bile duct [10].Distinction of high-grade dysplasia from invasive adenocarcinoma is usually not necessary on cytology and can be quite challenging. In the PSCSRPC it has been recommended that any BDB categorized as 'suspicious' may undergo FISH testing, which substantially improves diagnostic sensitivity without loss of specificity. Specimens with a 'suspicious' cytologic diagnosis and a positive FISH result may be referred for surgery. When material is not available for ancillary testing, review of the patient's overall clinical picture, endoscopy/imaging findings, and cytopathology results may help in arriving at a consensus diagnosis allowing referral for surgery or a decision to obtain additional testing [2].

Positive or Malignant:

Diagnosing malignancy in bile duct brushings is based on a constellation of cytologic features, and consideration of the overall picture- clinical presentation, radiology/ endoscopy findings, etc. Different studies have highlighted various key features for diagnosing malignancy in bile duct brushings. There is no standardized cut-off or major/ minor criteria for diagnosing malignancy/dysplasia in BDB. However, there are certain helpful features, which if applied carefully in conjunction with the overall clinical scenario, have a high specificity in diagnosis of malignant lesions. Any pathological diagnosis should be made in the context of sound clinical investigation including clinical history and the appropriate radiographic procedures. The referring clinician should have a clear understanding of the terminology used by the cytopathologist, and the pathologist should be experienced in the interpretation of biliary brushing specimens and be fully aware of the diagnostic pitfalls [12].

BDB from adenocarcinoma are usually hypercellular, and reveal *two distinct cell populations*, one of clearly benign and the other obviously atypical features (gradation of changes is a feature of reactive atypia) [23]. The cells show variable nuclear crowding (loss of honeycomb architecture)- ranging from mild nuclear overlap to more pronounced *three-dimensionality*, nuclear enlargement (*high n:c ratio*), *anisonucleosis* with a ≥1:4 variation in nuclear size within the same cell group, *irregular nuclear outlines* and heterogeneous chromatin distribution (mostly *coarse*, clumped and *hyperchromatic* but can be hypochromatic). Nucleoli may be prominent. Presence of *single malignant cells* in the background is an important indicator of malignancy [3, 10, 12-15]. Cell-in-cell arrangement and presence of mucin vacuoles have been described. Abnormal mitoses are a known malignant characteristic but are only rarely seen [14]. Necrosis and inflammation are non-specific findings that can also be

identified in benign samples. Renshaw et al, in their study, have stated that single malignant cells were not necessary for rendering 'malignant' diagnosis [3].Cohen *et al* showed that nuclear molding, chromatin clumping, and increased nuclear-cytoplasmic ratio were key cytologic features that were associated with malignancy. The presence of two of these features resulted in 83% sensitivity and 98% specificity for carcinoma detection [15]. Barr Fritcher et al found that abnormal single cells, nuclear membrane irregularity and enlargement were independently associated with malignancy [28]. Avadhani *et al* suggested that presence of ≥3 criteria (out of 11) was predictive of malignancy [14].

'Neoplastic' category features:

This is the most elusive category of all, and it hasn't been reported widely in literature mainly because the lesions in this category are very rarely seen in the bile ducts (even rarer on BDB) and cytomorphologically may appear banal. Neoplastic category lesions are mainly intraductal biliary tract neoplasms [categorized as *mucinous* (includes Intraductal papillary neoplasm of bile duct) or *non-mucinous* (Intraductal Oncocytic papillary neoplasm of the bile duct and Intraductal tubulo-papillary neoplasm of the bile duct)]. All these lesions are premalignant with potential to progress invasive tumors. *Intraductal papillary neoplasm of bile duct (IPNB)* is the bile duct equivalent of pancreatic Intraductal papillary mucinous neoplasm (IPMN) and shows papillary epithelial proliferation with delicate fibrovascular cores within dilated bile ducts. It displays a spectrum of dysplasia- ranging from low to high-grade, and has three epithelial subtypes- Gastric, Intestinal, Pancreatobiliary (most common) type. Pancreatobiliary subtype IPNB is high-grade, *by definition* [23]. *Intraductal Oncocytic papillary neoplasm of the bile duct (IOPNB)* is a high-grade papillary lesion with oncocytic cells, scant mucin, large nuclei, prominent eccentric nucleoli and punched out intercellular spaces. *Intraductal tubulo-papillary neoplasm of the bile duct (ITPNB)* is another high-grade lesion composed of back-to-back (cribriform) tubules lined by non-mucinous epithelium with high-grade atypia. It is predominantly a tubule forming neoplasm but may have a minor papillary component.

Ancillary Studies in Bile Duct Brushings

BDB cytology, even though very specific, suffers from a low sensitivity rate and its utility has been expanded by using molecular markers, immunostaining, and molecular testing. Search for improvements in diagnostic accuracy of BDB has led some to suggest triple testing (brush cytology, FISH and forceps biopsy) which has better accuracy (82% sensitivity, 100% specificity, 100% positive predictive value, and 87% negative predictive value) than brushing alone [29,30].

Immunohistochemistry can be useful in BDB cytology but lacks specificity. Immunostains need to be interpreted cautiously and results depend upon presence of specific mutation, timing/agent of fixation, etc. SMAD4 is probably the most reliable. Immunohistochemistry for p53 protein has been attempted but has yielded contradictory results, and is currently not recommended for routine diagnostic use [31]. S100P and p53 may stain reactive Ductal cells. Other markers have been used with variable sensitivity and specificity, but are not widely used (Maspin, Claudin-18, mCEA, IMP3, CD10, etc). Over 50% of biliary cancers showed a Maspin+/IMP3+/S100P+/pVHL − staining profile, and 20% showed a Maspin+/IMP3 − /S100P+/pVHL− profile in one study [32].

Fluorescence in situ hybridization (FISH) has shown promise as an adjunct in improving the sensitivity of cytology for the detection of malignant biliary strictures. In patients with negative cytology, FISH increases sensitivity while preserving specificity. A targeted FISH analysis of the atypical cells of interest allows for a more precise evaluation, even in paucicellular specimens. Use of cytology material for FISH does not require any alteration in specimen acquisition, only additional slides with cells of interest. It can be performed on liquid-based preparations as well as cell block materials [33]. However it poses technical and financial challenges [14]. Using the *UroVysion probe set* (Abbott Molecular Inc., Des Plaines, IL), has been shown to enhance the sensitivity of BDB cytology to 42.9% for detection of malignancy (higher than sensitivity of routine BDB cytology alone). The FISH probes detect aneuploidy in the centromeric regions of chromosomes 3, 7, and 17 and homozygous or heterozygous deletion of locus 9p21. The results of FISH testing need to be correlated with clinical and imaging findings in patients with PSC. Polysomy in the presence of a dominant stricture has a high positive predictive value for cholangiocarcinoma in patients with PSC [34]. *Pancreatobiliary FISH* includes probes for chromosomes 1q21 (MCL1), 7p12 (EGFR), 8q24 (MYC), and 9p21 (p16/CDKN2A). In comparison with the UroVysion FISH probe set or routine cytology, the sensitivity of Pancreatobiliary FISH (64.7%) was significantly higher (than the UroVysion probes (45.9%) ($P < .001$) or routine cytology analysis (18.8%) ($P < .001$), but all three methods have similar specificity (92.9%, 90.8%, and 100.0% respectively). Factors significantly associated with detection of carcinoma, in adjusted analyses, included detection of polysomy by pancreatobiliary FISH ($P < .001$), a mass by cross-sectional imaging ($P < .001$), cancer cells

by routine cytology (overall P = .003), as well as absence of primary sclerosing cholangitis (P = .011) [35].

Targeted next-generation sequencing (NGS) when combined with cytology, has been shown to have a sensitivity of 85% for the detection of malignancy in BDB- but on its own, NGS has sensitivity of 44%. It has a specificity of 96% [35]. NGS has shown driver mutations involving KRAS (90%), TP53 (60%), SMAD4 (25%), and CDKN2A (17%). Some alterations are actionable/therapeutic, including fusions involving ALK, NRG1, NTRK, ROS1 or mutations involving BRAF, BRCA1/2, HER2, KRAS, PALB2.

Conclusion

Endobiliary brush cytology is an established tool for cytologic evaluation of bile duct strictures and evaluation of dysplasia in patients with PSC. Though this method suffers from a low sensitivity, it has high specificity for lesions categorized as 'Suspicious' or 'Malignant'. There is a real need for the development of a uniform, standard reporting system which caters to biliary brushing specimens in order to determine more accurately the sensitivity and the risk of malignancy for the different diagnostic categories. Cytologic diagnosis of malignancy should be made in conjunction with the patient's overall clinical, radiologic, and endoscopic features. Cytopathologist should be aware of diagnostic pitfalls and with communicate with the referring clinician to ensure clear understanding of the diagnostic terminology used. Use of ancillary studies is important, especially in the atypical/suspicious lesions.

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Competing Interests

None

List of commonly used abbreviations in the article:

ERCP: Endoscopic Retrograde Cholangiopancreatography

PSCSRPC: Papanicolaou Society of Cytopathology System for Reporting Pancreaticobiliary Cytology

PSC: Primary Sclerosing Cholangitis

PTHA: Percutaneous Trans-Hepatic Aspiration

BDA: ERCP-guided Bile Duct Aspiration

BDB: ERCP guided Bile Duct Brushing

FISH: Fluorescence in-situ hybridization

BilIN: Biliary Intraepithelial Neoplasia

CBD: Common Bile Duct

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