Original Article

Whole slide image with image analysis of atypical bile duct brushing: Quantitative features predictive of malignancy

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Abstract

Background: Whole slide images (WSIs) involve digitally capturing glass slides for microscopic computer-based viewing and these are amenable to quantitative image analysis. Bile duct (BD) brushing can show morphologic features that are categorized as indeterminate for malignancy. The study aims to evaluate quantitative morphologic features of atypical categories of BD brushing by WSI analysis for the identification of criteria predictive of malignancy. **Materials and Methods:** Over a 3-year period, BD brush specimens with indeterminate diagnostic categorization (atypical to suspicious) were subjected to WSI analysis. Ten well-visualized groups with morphologic atypical features were selected per case and had the quantitative analysis performed for group area, individual nuclear area, the number of nuclei per group, N: C ratio and nuclear size differential. **Results:** There were 28 cases identified with 17 atypical and 11 suspicious. The average nuclear area was 63.7 μm^2 for atypical and 80.1 μm^2 for suspicious (+difference 16.4 μm^2; P = 0.002). The nuclear size differential was 69.7 μm^2 for atypical and 88.4 μm^2 for suspicious (+difference 18.8 μm^2; P = 0.009). An average nuclear area >70 μm^2 had a 3.2 risk ratio for suspicious categorization. **Conclusion:** The quantitative criteria findings as measured by image analysis on WSI showed that cases categorized as suspicious had more nuclear size pleomorphism (+18.8 μm^2) and larger nuclei (+16.4 μm^2) than those categorized as atypical. WSI with morphologic image analysis can demonstrate quantitative statistically significant differences between atypical and suspicious BD brushings and provide objective criteria that support the diagnosis of carcinoma.

Key words: Adenocarcinoma, bile duct, cytology image analysis, whole slide image

INTRODUCTION

Whole slide images (WSIs) provide a method for digitally capturing pathologic slides and the platform offers a variety of options and uses to extend and expand the use and analysis of the traditional morphologic pathologic material. In addition to educational, archival and remote viewing, WSIs can be subject to image analysis. Instead of select static images, an entire slide

or groups of slides can be studied by the methodology. A variety of vendors offer robust software solutions, which measure defined morphologic features in an automated or semi-automated manner. These can measure traditional staining morphology (H & E staining, Papanicolaou staining, etc.) and quantitative and qualitative features of immunohistochemistry stains (human epidermal growth factor receptor 2 [HER-2]/neu).[1,2]

In cytopathology, morphologic specimen interpretation serves as the bedrock for specimen interpretation and diagnosis. This interpretation is informed by observational experience, knowledge of expected and observed features and subjective individual judgment. Image analysis offers an objective tool by which measurement of morphologic features can be performed.

In cytopathology, bile duct (BD) brushing is commonly utilized to evaluate pancreatobiliary strictures. Patients who present with biliary stricture can have a variety of possible etiologies including benign inflammatory conditions, intrabiliary lithiasis (stones) or carcinoma (cholangiocarcinoma/adenocarcinoma). The clinical features are not always determinative, and a BD brush cytology can help to stratify patients in the context of other clinical and radiographic findings. BD brush specimens are challenging to interpret since they often demonstrate an inflammatory reactive background due to the local effects of stricture and are often low to intermediate cellularity. As a result, atypical and suspicious categories are commonly reported. Indeterminate diagnostic categories are not as helpful as benign and malignant diagnostic diagnoses for clinical management.

The aim of the study was to evaluate biopsy proven carcinoma with indeterminate BD brush cytology specimens (atypical and suspicious categories) with WSI analysis in order to identify objective morphologic criteria that might further stratify risk of malignancy.

MATERIALS AND METHODS

Over a 3-year consecutive period, the pathology database was searched for BD brush specimens with indeterminate diagnostic categorization. These included "atypical" or "suspicious" in the cytopathologic report during the course of the routine patient evaluation. Cases reported as benign, and adenocarcinoma/malignant/positive were excluded. For this indeterminate group, the corresponding pathology database was cross-referenced, and specimens with a corresponding histologic confirmation of carcinoma were selected. Only cases with an indeterminate cytopathologic diagnosis (atypical/suspicious) and histologic diagnosis of carcinoma were included in the study group. Pathology reports were collected and patient demographics and

data were reviewed. BD brush specimens are processed from a liquid fixative (PreservCyt™, Hologic Corporation) and a single non-Gyn Filter ThinPrep™ slide (Hologic Corporation) is made. It is stained by a traditional Papanicolaou staining method and interpreted by a pathologist. The individual slides from each individual case were placed in an Aperio® Scanscope XT 120 (Leica Corporation) slide scanner and using the ×20 (doubler) scanning process, a WSI was created.

Within the Aperio® Spectrum™ software, an individual case was opened in the ScanScope/ImageScope™ software (v10.2.2.2352 Leica Biosystems Inc., Buffalo Grove, Illinois, USA). The entire WSI was available for review. Using the scroll and zoom in/out capability, the entire slide was systematically reviewed by a single operator without knowledge of original categorization or patient outcome. Postprocessing image adjustment built into the software (brightness/contrast) was adjusted to improve visualization of three-dimensional groups and better identify individual nuclear outlines within a group. The operator was instructed to identify the most cytologically abnormal groups that could be well-visualized and the 10 best were manually selected. These were identified and the outer limit of each individual group was delineated using the "pen tool" which is available in the ImageScope™ software. The operator identified the outer limits of the group cytoplasm and completely encircled the entire group. Groups with benign features such as small orderly monomorphic nuclei and flat sheets were not selected. Instances where the groups were difficult to visualize, or other characteristics were not clearly apparent were not selected; the operator used their best judgment based on the individual group's morphology. Exclusion criteria included single epithelial cells, groups of indeterminate origin (secondary to crush, poor preservation, or staining), and nonepithelial cells (neutrophils, singly, and in clusters). Ten groups were selected and then a manual process was used to delineate the outside margins of the group and individual nuclei [Figure 1]. Within the 10 selected individual groups, the individual nuclei were manually selected

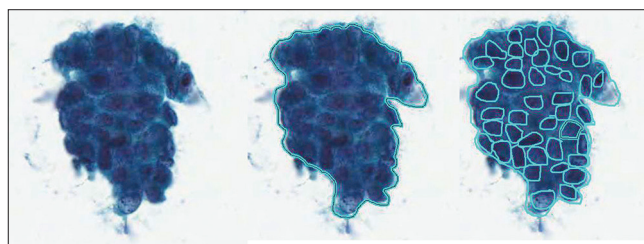**Figure 1: Example of the group delineation process for a whole slide image. Once the group has been identified, the ImageScope™ software pen tool is used to identify the outer margins of the group as noted in the middle panel. Then the individual nuclei are delineated, as shown completed in the right-sided panel. (Aperio™ whole slide image screen capture with pen tool delineation, Papanicolaou-stained ThinPrep™ slide)**

and delineated by the "pen tool" [Figure 2]. These qualified and delineated "pen tool" groups were recorded within the ImageScope™ software in the "annotations" dialog box. Within the "annotations" dialog box, each individual group is given a "region" designation under a single "layer" category [Figure 3]. Ten of these were
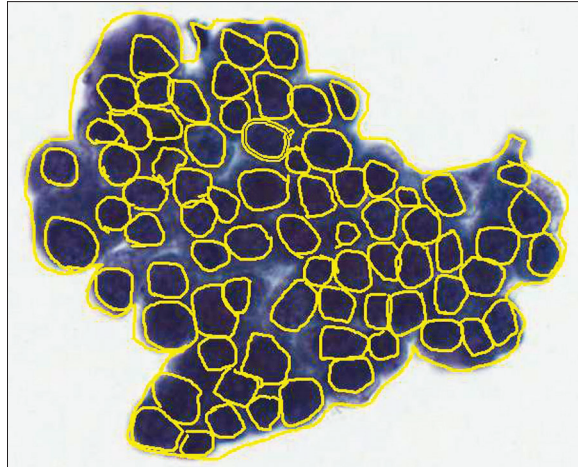

Figure 2: An Individual group with the pen tool delineation. The outside of the group is entirely circled. Each individual nuclei within the group are delineated. (Aperio™ whole slide image screen capture with pen tool delineation, Papanicolaou-stained ThinPrep™ slide)

Figure 3: ImageScope™ Annotations Dialogue box with data for an individual group. The first line is the entire single group delineation. The other region lines are individual nuclei. (Screen capture, Aperio ImageScope™ Software, Leica Corporation)

recorded for each analyzed group in individual cases. At the completion of the process, the individual region designation allowed the operator to review each group by clicking on the "region" which then takes the view screen to a high power encompassing view of the specific group. The entire manual process of group and nuclei selection took an average of 15–20 min/WSI. After completing the process of delineating groups of interest for the WSI, the objective quantitative image analysis process was performed. The manual process of delineating the groups of interest was performed by a single operator (RCW).

Each individual group was quantitatively analyzed for the number of total nuclei, individual nuclear area (μm^2), and total group area (μm^2). Based on the known magnification of the images by the software program, these are calculated directly. From this directly measured data, the average number of nuclei per group, average nuclear to cytoplasmic (N/C) ratio per group and range of nuclear area (nuclear size differential) per group were calculated.

After the analysis, the cumulative WSI "layer" region result is exported by the ImageScope™ software to a Microsoft Excel™ spreadsheet file which has an individual line for each delineated and measured region. A large amount of data is recorded, only some of which is pertinent and utilized. The directly measured data fields used are: Group area (μm^2), total nuclei per group (numeric), and individual nuclear size (μm^2) [Figure 4]. From the Excel™ spreadsheet, the measured group size is known, and the total nuclear area calculated (total nuclei area added per group), and then the (N/C) ratio is calculated by dividing the measured total nuclear area by the measured group area (nuclear area/group area). The overall calculated average N/C ratio for the individual WSI is obtained by taking the added total of each N/C ratio and dividing it by the total group number (total of all N/C ratio/total group number). The range or difference in nuclear size per each individual group was measured, and then the average nuclear size differential per WSI was calculated.

Once the calculations were completed independently and in a blinded fashion, the individual cases were correlated with the pathologic reports. The BD brush WSI cases

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Group	1	2	3	4	5	6	7	8	9	10 SUMM		Average
2	Total nuclei per individual group	7	11	4	9	2	13	30	2	29	9	116	11.6
3													
4	Area of nucleus: average	87.04	61.6	93.225	39.28889	85.3	51.45385	50.65667	110.35	60.27931	71.14444	710.3410136	71.0341014
5													
6	Area of nucleus: differential	103.2	57.4	119.5	47.2	45.4	58.4	76.7	81.3	156.1	69	814.2	81.42
7													
8	NC ratio: average	0.62	0.5586	0.54518	0.321747	0.523313	0.443862	0.331234	0.586968	0.672605	0.420696	5.024052617	0.50240526
9													
10													

Case Totals ╱ Group 1 ╱ Group 2 ╱ Group 3 ╱ Group4 ╱ Group5 ╱ Group6 ╱ Group7 ╱ Group8 ╱ Group9 ╱ Group10

Figure 4: Microsoft Excel spreadsheet with individual case data. Ten groups were analyzed per case, and these are each included and an average for total nuclei per group, the average area of nuclei, an average differential of the nuclear area and average nuclear to the cytoplasmic ratio calculated. (Screen capture, Microsoft Excel™ spreadsheet, Office 2011)

were categorized into "atypical" and "suspicious" groups based on the original pathologic classification. Data analysis on the Microsoft® Excel file was performed by the Excel™ software program on individual cases and for analysis of group categories and associated measured outcomes. The *P* values were calculated using standard calculation formulas and independent two sample *t*-test calculations (IBM Corporation, SPSS Statistics, version 21 Armonk, New York. USA). A result was considered statistically significant if $P < 0.05$. A relative risk ratio was calculated, and it was intended to provide information about the image analysis cut-off values. It is not a standard risk ratio of malignancy. The risk ratio is calculated utilizing the cases that were equal to or greater than the cut-off values as a true positive result. This is intended to help stratify the image analysis features within the context of these indeterminate cytopathology brush classifications where patients were determined to have carcinoma. The study had the approval of the Institutional Review Board.

RESULTS

Twenty-eight patients were identified. All patients were originally categorized as either "atypical" or "suspicious" by the pathologist. BD brush cases were interpreted by a mixture of pathologists during the course of routine clinical service. No attempts were made to further subtype or reclassify the initial categorization. Histologic and surgical excision for all cases confirmed the presence of carcinoma. This was performed from a variety of methods that included forceps biopsy at the time of BD brush specimen collection and subsequent staging and Whipple procedures.

The image analysis results are presented in Table 1. As per the image analysis method described in the materials and methods section, a variety of measured and calculated endpoints were collected. Based on the original cytopathology report, the individual WSI cases were stratified into atypical and suspicious end-point groups. WSI of the BD brushing of the atypical cohort class (17) showed a range of results for each individual case including average total nuclei per group of 12.97 (range: 5.2–29), average area of nucleus 63.59 μm² (range: 35.6–86.9 μm²), average nuclear area differential 69.7 μm² (range: 36.3–110.7 μm²), and an average N/C ratio range

of 0.52 (range: 0.38–0.66). WSI of the BD brushing of the suspicious cohort class (11) showed a range of results for each individual case including average total nuclei per group of 12.28 (range: 7.3–18), average area of nucleus 80.05 μm² (range: 70.4–109.6 um²), average nuclear area differential 88.4 μm² (range: 67.7–107.6 um²), and an average N/C ratio range of 0.53 (range: 0.47–0.66).

The image analysis comparison data is presented in Table 2. The average total nuclei per group showed a 0.69 differential ($P = 0.387$) (12.97 for atypical and 12.28 for suspicious). The average nuclear area showed a 16.46 μm² differential ($P = 0.002$) (63.59 μm² for atypical and 80.05 μm² for suspicious). The average area of nucleus differential size was 18.7 μm² ($P = 0.009$) (69.7 μm² for atypical and 88.4 μm² for suspicious). The average N/C ratio differential was 0.008 ($P = 0.387$) (0.523 for atypical and 0.531 for suspicious). A $P \leq 0.05$ was considered statistically significant.

Data points and thresholds were analyzed and stratified within the cohorts [Table 3]. When categorizing the cases with an average nuclear area >70 μm², these included all of the suspicious cases (11/11) and 5 of the

Table 1: Atypical bile duct brush WSI: Image analysis results

	Image analysis data
Atypical (17)	
Total nuclei per group, range	5.2 to 29
Total nuclei per group, average	12.97
Area of nucleus, average, range	35.6 to 86.9 μm²
Area of nucleus, average	63.59 μm²
Area of nucleus: differential, range	36.3 to 110.7 μm²
Area of nucleus: differential, average	69.7 μm²
N/C ratio, range	0.38 to 0.66
N/C ratio, average	0.52
Suspicious (11)	
Total nuclei per group, range	7.3 to18
Total nuclei per group, average	12.28
Area of nucleus, average, range	70.4 to 109.6 μm²
Area of nucleus, average	80.05 μm²
Area of nucleus: differential, range	67.7 to 107.6 μm²
Area of nucleus: differential, average	88.4 μm²
N/C ratio, range	0.47 to 0.66
N/C ratio, average	0.53

N/C: Nucleus to cytoplasm

Table 2: Atypical bile duct brush WSI: Comparison data

WSI image analysis data points	Atypical	Suspicious	Differential	*P* value
Total nuclei per group, average	12.97	12.28	0.69	0.387
Area of nucleus, average	63.59 μm²	80.05 μm²	−16.46 μm²	0.002
Area of nucleus: differential, average	69.7 μm²	88.4 μm²	−18.7 μm²	0.009
N/C ratio, average	0.523	0.531	0.008	0.387

N/C: Nucleus to cytoplasm; *P* value ≤0.05 statistically significant

Table 3: Atypical bile duct brush WSI: Risk ratio

Data points	Atypical	Suspicious	Risk ratio	*P* value
Nuclear area greater than 70 μm^{2a}	5/17	11/11	3.2	0.0015
Nuclear differential greater than 75 μm^{2b}	6/17	9/11	2.6	0.0094

a: Nuclear area average per individual WSI case, b: Area of nucleus differential averaged per individual WSI case, *P* value ≤0.05 statistically significant

atypical cases (5/17). When categorizing the cases with a nuclear differential >75 μm^2, these included 9 suspicious cases (9/11) and 6 of the atypical cases (6/17). Using the relative risk ratio formula, a nuclear area >70 μm^2 had a risk ratio of 3.2 ($P = 0.0015$). Using the relative risk ratio formula, a nuclear area differential >75 μm^2 had a risk ratio of 2.6 ($P = 0.0094$).

DISCUSSION

WSI is becoming more widely available, and the technology offers a method to digitize traditionally prepared pathology slides and smears. There are many practical and theoretical advantages to utilizing a digital pathology specimen. WSI are gaining wide use in educational activities, specimen archival applications, remote viewing uses and second opinion/consultations.[3-6] One advantage to WSI of pathology slides includes the ability to assess and evaluate the digitized information by image analysis. This includes currently Food and Drug Administration approved algorithm for image analysis of HER-2/neu immunohistochemistry.[1,2] The method is applicable for histologic and cytopathologic preparations. For cytopathology, WSI technology can digitize the variety of specimen slides prepared for evaluation including cytospin-concentration techniques, aspirate smears, liquid-based slides, brushing specimens, and cell blocks; each of which include a range of staining methods including Papanicolaou, modified Giemsa, and H & E. Once digitized, the image analysis toolbox is available to objectively analyze and evaluate the cellular elements present in cytopathology specimens. WSI and image analysis technologies provide the opportunity to examine an entire slide or group of slides. As a platform, these can work together as either an automated or semi-automated workflow process.

Pathology diagnosis is based on morphologic observation of glass slides utilizing long standing traditional tools of light microscopy. For cytopathology, the morphologic judgment classifies specimens with a precise diagnosis. Due to innate features of sampling methods and clinical circumstances, there are cytopathology specimens that have an indeterminate classification, outside the benign or malignant groups. BD brushing specimens are one area where definitive classification is not always possible. The morphologic diagnostic challenges of BD brushing are well described and well-known by those who practice cytopathology and clinicians who rely on the testing to help manage their patients.[7-11] In brief, some of the factors are worth discussing. BD strictures are often in anatomic locations that are difficult to reach by endoscopy. To obtain cellular elements, a BD brushing sample is frequently collected. Traditionally, the brush is introduced into the area of stricture and moved back and forth in the area of stricture to dislodge and collect cells. These areas of stricture are often narrow and have associated reactive glandular epithelial cells and surrounding fibrosis, whether it is a benign inflammatory or neoplastic stricture. The narrowed region and fibrosis can contribute to a sparsely cellular sample. The combination of a sparsely cellular sample and background reactive epithelial cells often contributes to an indeterminate diagnostic classification. These categories tend to include "atypical epithelial cells" and "suspicious for carcinoma." The criteria utilized for these diagnostic reporting groups are not standardized and rests on the judgment and experience of the interpreting pathologist. In general, a negative categorization and malignant categorization have good reproducibility and both negative predictive value and positive predictive value across different studies. The indeterminate criteria are not as reproducible or have strong statistical certainty related to patient outcome.[9]

Therein lies the value in utilizing an objective measurement method to assist in stratifying and further classifying those indeterminate diagnostic categories which can be present in cytopathology, and specifically in BD brush cytology. Bridging the observational judgment of malignancy assessment by an individual pathologist with an adjunctive objective ancillary testing system has the potential to assist in diagnostic clarification and provide further information for the clinician in managing their patient. For patients with a BD brush diagnosed as either atypical or suspicious for malignancy, can image analysis data point performed on a WSI provide specific metrics, which can assist in further stratifying risk of malignancy. A variety of authors has described utilizing DNA image cytometry on BD cytology brush specimens using a variety of methods and data points.[9-14] Ours is the first to apply an image analysis algorithm to BD brush WSI and the technique has only been infrequently described in cytopathology.[15]

In this study, there were a number of similarities and differences between the atypical and suspicious cohorts. There was no statistical difference between average total nuclei per group (12.97 vs. 12.28). Theoretically, adenocarcinoma in BD brushing has been observed to have more single atypical and malignant cells, which

represent a loss of cellular cohesion in carcinoma in contrast to a reparative epithelial process where the groups are larger, hold together and lack single atypical cells. There was no statistical difference between the average N/C ratio (0.523 vs. 0.531). Adenocarcinoma can be recognized by alteration in the cellular N/C ratio, where benign cells retain a moderate to abundant volume of cytoplasm in contrast to malignant cells that have less differentiation and less cytoplasm imparting a higher N/C ratio.

There were differences observed, which involved the average nuclear area and an average of the nuclear size differential. Adenocarcinoma in BD brush often show nuclear enlargement. The "two cell population" of malignant cells and benign cells intermixed in a BD brush is morphologically observed at a medium power and largely imparted by nuclear size with malignant cells being larger than benign BD nuclei. The average area of the nucleus was statistically different with 63.59 μm^2 for atypical compared to 80.05 μm^2 for the suspicious cohort (differential of 16.46 μm^2) ($P = 0.002$). This difference in nuclear size average likely accounts for the difference in categorization between atypical and suspicious. With relatively smaller nuclei in the atypical cohort, the pathologist likely had less confidence of a malignant category and, therefore, did not place these cases into the suspicious category.

A key feature commonly emphasized in the diagnosis of adenocarcinoma of the pancreatobiliary system is anisonucleosis. This is a variation in the size of individual nuclei within a group and generally quantitated by observation is multiple of size ($\times 1$ times, $\times 2$ times, $\times 3$ times, etc.). Observational experience has shown that benign BD epithelial cells will show virtually no difference and reactive reparative change tends to be more modest with 1–2 times variation. Adenocarcinoma tends to show a more pronounced variation of 3–4 times.[16] The average nuclear area differential was statistically different with 69.7 μm^2 for the atypical and 88.4 μm^2 for the suspicious cohort (differential of 18.7 μm^2) ($P = 0.009$). The more pronounced size differential measured in the suspicious cohort likely contributed to the pathologist degree of concern resulting in a suspicious category, instead of an atypical category.

Some of the other observational features of malignancy are more difficult to objectively quantify by image analysis. Focal crowding and overlap with the cellular disorder can be subtle and require more complex image analysis. Nuclear membrane irregularities and chromatin structure/pattern are important diagnostic features in the evaluation of malignancy. These data points are more difficult to quantify and are beyond the scope of most core image analysis software capabilities.

There is always variability of measures within a case and between cases when measured individually and in aggregate. When a large amount of information and data points are collected and aggregated, it can help in classification to set predetermined cut points to help interpret the findings. Within this cohort, certain patterns and grouping of data was observed and based on this stratification data points were assigned within the two classes that had statistical significance. For the average nuclear area, a data point of 70 μm^2 was set, and 5/17 atypical cases and 11/11 suspicious cases were above this number. Using a risk ratio calculation, an individual case above the data point had a risk ratio of 3.2. For the average nuclear differential, a data point of 75 μm^2 was set, and 6/17 atypical cases and 9/11 suspicious cases were above this number. Using a risk ratio calculation, an individual case above the data point had a risk ratio of 2.6. The assignment of stratification data and risk ratio has the potential to add additional information to the observational diagnostic interpretation, and in turn add to the information a clinician has in the evaluation of a biliary stricture that also includes clinical and radiographic findings.

CONCLUSION

The quantitative criteria findings as measured by image analysis on WSI showed that cases categorized as suspicious had more nuclear size pleomorphism (+18.8 μm^2) and larger nuclei (+16.4 μm^2) than those categorized as atypical. WSI and morphologic image analysis can demonstrate quantitative statistically significant differences between atypical and suspicious BD brushings and provide objective criteria that support the diagnosis of carcinoma.

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Conflicts of Interest
There are no conflicts of interest.

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