Assessment of Nuclear Nanomorphology Marker to Improve the Detection of Malignancy From Bile Duct Biopsy Specimens

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ABSTRACT

Objectives: The accurate diagnosis of malignancy from small bile duct biopsy specimens is often challenging. This proof-of-concept study assessed the feasibility of a novel optical technology, spatial-domain low-coherence quantitative phase microscopy (SL-QPM), that assesses nanoscale structural alterations in epithelial nuclei for improving the diagnosis of malignancy in bile duct biopsy specimens.

Methods: The SL-QPM analysis was performed on standard histology specimens of bile duct biopsy specimens from 45 patients. We analyzed normal cells with benign follow-up, histologically normal cells with pancreaticobiliary malignancy, and malignant epithelial cells.

Results: The SL-QPM–derived nuclear nanomorphology marker can not only distinguish benign and malignant epithelial cells but can also detect features of malignancy in those cells normal by light microscopy with a discriminatory accuracy of 0.90. When combining pathology with SL-QPM, the sensitivity is improved to 88.5% from 65.4% of conventional pathology, while maintaining 100% specificity.

Conclusions: SL-QPM–derived nuclear nanomorphology markers represent a novel approach for detecting malignancy from histologically normal-appearing epithelial cells, with potential as an adjunctive test in patients with negative or inconclusive pathologic diagnosis on bile duct biopsy specimens.

Analysis of bile duct biopsy specimens is critical for the clinical management of patients with indeterminate biliary strictures. While conventional histologic diagnosis has a relatively high specificity (approaching 100%) for malignancy, the sensitivity remains suboptimal, often ranging from 40% to 75%.1-6 The biggest challenge in detecting malignancy on bile duct biopsy specimens is identifying an adequate number of cells that meet the diagnostic criteria of malignancy. The anatomy of the biliary tract makes it difficult to directly visualize strictures as well as the biopsy site, leading to small tissue fragment size and potential sampling error. Therefore, the sampled epithelial cells may not be from the stricture but rather from adjacent biliary epithelial cells. The small tissue fragment size often makes the identification of tissue architectural features more difficult. Furthermore, the identification of malignant cells is also confounded by the presence of inflammation. Because of the limitations of the tissue characteristics, an inconclusive diagnosis of atypical or suspicious may be rendered. As a result, it often takes multiple biopsies and several months to arrive at the diagnosis of malignancy.

The standard pathologic diagnosis is based on identifying morphologic features characteristic of malignancy using a conventional light microscope. The resolution of light microscopy is limited by diffraction, which can only detect structural alterations at the scale of around 1 μm. Our group has developed a novel optical microscopy technique—spatial-domain low-coherence quantitative phase microscopy (SL-QPM)—that is capable of detecting nanoscale nuclear morphology, or the structural changes in the cell nucleus as small as 1 nm, which is about 1,000 times more sensitive than what conventional bright-field light microscopy detects.7,8 The SL-QPM uses the inherent configuration of tissue sandwiched
between a glass slide and coverslip to construct a common-path light interferometry condition, which provides ultra-high sensitivity to detect minute structural changes down to 1 nm while maintaining the required stability for clinical sample analysis. Importantly, this technique is suitable for analyzing nuclear nanomorphology on a standard histology slide without any modification, thus making this approach simple and cost-effective. We have previously shown the great potential of this technique in detecting the presence of various types of malignancy, even from those epithelial cells that appear normal to pathologists by light microscopy.9-14

In this proof-of-concept study, we explored the feasibility of identifying an SL-QPM–derived nanomorphology marker in the epithelial cell nuclei of bile duct biopsy specimens. We hypothesized that cells distant from the stricture or in the early course of cancer development may display subtle nanoscale structural changes (as detected by SL-QPM) that are undetectable by conventional light microscopy. Our ultimate goal is to incorporate this novel technique into the clinical diagnostic workflow of bile duct tissue biopsy specimens, thereby reducing the number of inconclusive atypical or suspicious diagnoses and the number of false-negative results.

Materials and Methods

Specimens

All studies were performed with the approval of the institutional review board at the University of Pittsburgh. Archived histology slides of bile duct biopsy specimens at the University of Pittsburgh Medical Center that were obtained by either endoscopic retrograde pancreatography or cholangioscopy (SpyGlass, Boston Scientific Corporation, Natick, MA) for possible biliary tract malignancy were studied. All bile duct biopsy specimens were subjected to a routine protocol that consisted of cutting two H&E-stained slides, six unstained slides, and then one additional H&E slide during initial processing. Table II shows the characteristics and follow-up for the 45 patients in this study. The pathologic diagnosis of each bile duct biopsy specimen was confirmed by two gastrointestinal pathologists (D.J.H. and A.M.K.). The final diagnosis was based on concurrent or subsequent clinical, radiographic, and/or pathologic information.

SL-QPM

A detailed description of the SL-QPM instrument and data analysis algorithms has been reported elsewhere. Briefly, SL-QPM uses a low-coherence white-light source Xe-arc lamp (Newport Corporation, Newport, CT) that is collimated by a 4f imaging system and focused onto the sample by an objective (NA = 0.4). The reflectance-mode image is collected by a scanning imaging spectrograph (Princeton Instrument, Acton, MA) coupled with a CCD camera (Andor Technology, Belfast, Northern Ireland). The CCD camera records a three-dimensional intensity cube $I(x, y, k)$, where $x$ and $y$ represent the spatial position of each pixel in the microscopic image and $k$ represents the wave number. The transmission optics is also used to record a conventional histologic image.

The nanoscale structural information is derived from the common-path interferometry configuration inherent in the original histology slide itself. The reflected light from the glass-tissue interface serves as the reference wave, and the backscattered light from the cell on the histology slide serves as the sample waves, thus eliminating the multiple noise sources of traditional interferometry, achieving a superior sensitivity of around 1 nm.7,8

The samples used for SL-QPM analysis were the same H&E-stained slides that were used for the clinical diagnosis; no special preparation of the tissue was needed. The pathologist (D.J.H. or A.M.K.) marked epithelial cells of interest on the histology slides for SL-QPM analysis. The person who performed the optical measurements was blinded to the pathologic diagnosis and the final follow-up diagnosis.

We have extensively evaluated the contribution of various confounding factors and have addressed the variation in the staining level, variation of tissue section thickness, number of cells to be analyzed, and interuser variability and found that the variation can be minimized to be within the system sensitivity.8,11,12

Analysis of the Nuclear Nanomorphology Marker From the Cell Nucleus

The recorded spectroscopic image $I(x, y, k)$ from each pixel of the microscopic image was mathematically transformed to obtain a two-dimensional optical path length difference (OPD) map from the cell nucleus, which was subsequently used to quantify the nuclear nanomorphology marker, as previously described. In brief, the images of cell nuclei were first segmented. The reflectance spectrum $I(x, y, k)$ from each pixel $(x, y)$ of each nucleus image was then normalized by the spectral profile of the optical system to account for the wavelength-dependent response of the light source and all the optical components. After taking the discrete Fourier transform of the collected signal $I(x, y, k)$, the OPD at a fixed optical path length of interest was obtained using the following equation:

$$\text{OPD}(x, y) = \frac{\lambda_0}{2 \times 2\pi} \arctan \left( \frac{\text{Im}(\rho(z_{\text{opt}})k)}{\text{Re}(\rho(z_{\text{opt}})k)} \right)$$

where $\lambda_0$ is the source central wavelength ($\lambda_0 = 550$ nm), $z_{\text{opt}}$ is the fixed optical depth location, and Im and Re denote the imaginary and real parts of the complex value $\rho(z_{\text{opt}})$, respectively. Note that the factor of 2 accounts for the double OPD due to the reflection configuration. Then we also obtain a transmission image to measure the amount of stain-induced OPD, which is used to account for the effect of variation using a validated staining correction model.12 As a result, we obtain a final OPD map.
Statistical Analysis

To quantify the statistical nanomorphology characteristics of the OPD map in the nucleus, we used the mean OPD \( \langle OPD \rangle \) from each cell nucleus as the representative statistical nuclear nanomorphology marker. Then we analyzed this marker for approximately 30 to 40 epithelial cell nuclei and obtained their mean value of \( \langle OPD \rangle \) as the characteristic value for an individual patient, denoted as \( \langle \langle OPD \rangle \rangle_p \). The statistical comparison between two patient groups was obtained using the \( t \) test at a 95% confidence interval, and two-sided \( P \) values were used for all analyses. A \( P \) value of .05 or less was considered statistically significant. We conducted a prediction model using the logistic regression of \( \langle \langle OPD \rangle \rangle_p \). The receiver operating characteristic (ROC) curves were calculated using the logistic regression model and leave-one-out cross-validation.

Results

Categorization of Specimens for SL-QPM Analysis

We analyzed three groups of epithelial cell nuclei on the histology slides of bile duct biopsy specimens from a total of 45 patients: (1) benign group (n = 19), including cells from
patients who had a final diagnosis of benign; (2) uninvolved group (n = 18), including cells from patients who had a final follow-up diagnosis of malignancy, but only those cell nuclei labeled as benign by the pathologists were analyzed (some cases that also had malignant cells in the same biopsy specimen are included in this group); and (3) malignant group (n = 19), including malignant cells from patients who had a final follow-up diagnosis of malignancy. Representative images of benign, reactive/inflammatory, atypical/suspicious, and malignant are shown in **Image 1**. All patients included in this study had clinical findings of a biliary stricture, lesion, or obstructive jaundice. Four of the 19 patients with benign or reactive/inflammatory diagnoses had primary sclerosing cholangitis, and one patient had bile duct stones with a possible stricture. Four of the 19 patients had active inflammation while three had chronic inflammation; one had both active and chronic inflammation. No difference was observed in the utilization of additional levels or immunohistochemistry between patients with benign or malignant diagnoses. The most common immunohistochemical stains ordered during the initial clinical workup were p53, Ki-67, and pankeratin (Table 1).

**OPD Map**

**Image 2** shows the representative histologic images and the corresponding pseudo-color OPD maps from the cell nuclei of benign, uninvolved, and malignant groups. The OPD maps of cell nuclei exhibit a distinct spatial distribution and overall OPD values between benign and malignant groups. More important, the OPD maps of the cell nuclei from the
Representative histologic images (H&E, ×145) and corresponding OPD maps of the (A) benign group (histologically normal cells from patients with a benign final diagnosis), (B) uninvolved group (histologically normal cells from patients with a malignant final diagnosis), and (C) malignant group (histologically malignant cells). Color bar represents the magnitude of optical path length in micrometers.
uninvolved group (normal-appearing cells from patients with malignancy) are more similar to the OPD maps of malignant cells than are benign cells (Image 2). This result suggests that the OPD maps present some cancer-like nanomorphology signatures in the normal-appearing cells from patients with malignancy that cannot be easily appreciated by conventional light microscopy.

Nuclear Nanomorphology Markers

To quantify the signatures reflected in these OPD maps of cell nuclei, we performed statistical analysis by extracting a simple nanomorphology marker, average OPD $<\text{OPD}>$ from the OPD map, for each cell nucleus. To obtain a characteristic marker for each patient, we get the mean value of nuclear $<\text{OPD}>$ for around 30 to 40 epithelial cell nuclei for benign and uninvolved groups and for all malignant cell nuclei present on the histology slides for the malignant group, denoted as $<\text{OPD}>_p$. As shown in Figure 1, the nuclear nanomorphology marker $<\text{OPD}>_p$ of the malignant group (mean value = 0.1574 μm) is significantly increased compared with that of the benign group (mean value = 0.1495 μm) ($P = 0.00015$), supporting that this marker distinguishes malignant from normal cells. Importantly, this nuclear nanomorphology marker that identifies a nanoscale structural feature can even detect the presence of nearby malignancy from epithelial cells that appear normal under light microscopy, as evidenced by the statistically significant increase of $<\text{OPD}>_p$ in the uninvolved group—that is, histologically normal-appearing epithelial cells from patients with malignant strictures (mean value = 0.1584 μm) compared with that of the benign group (mean value = 0.1495 μm) ($P = 0.0004$). This technology appears to identify a common nanoscale structural feature in patients with malignancy regardless of the cell type studied (based on conventional light microscopy), since there was no statistically significant difference between the uninvolved group (mean value = 0.1574 μm, histologically normal-appearing cells) and the malignant group (mean value = 0.1584 μm, malignant cells) ($P = 0.71$).

Furthermore, to evaluate the utility of this technique in patients with inconclusive pathologic diagnoses on the bile duct biopsy specimens, we assessed the nuclear nanomorphology marker of $<\text{OPD}>_p$ in nine patients who had a pathologic diagnosis of atypical or suspicious on their bile duct biopsy specimens Figure 2. Even with this small number of inconclusive cases, the nuclear nanomorphology marker of $<\text{OPD}>_p$ shows statistical significance between those patients who had a follow-up malignant diagnosis and those who had a follow-up benign diagnosis ($P = 0.03$). The mean follow-up for patients who had atypical/suspicious diagnosis but were considered to have benign disease in follow-up was 652 days (range, 0-1,233 days). These results, although preliminary, provide further support that the nuclear nanomorphology marker can distinguish between benign and malignant strictures.

Performance Characteristics

To evaluate the performance characteristics of the nuclear nanomorphology marker from histologically normal-appearing cells for potential clinical use to improve the diagnostic accuracy of malignancy from bile duct biopsy specimens, we constructed ROC curves based on a logistic regression model. Figure 3 shows the ROC curve to detect patients with malignancy simply by analyzing one nuclear nanomorphology marker ($<\text{OPD}>_p$) from histologically normal-appearing cells. The discriminant power of the model, assessed by means of the area under the ROC curve (AUROC), is 0.90. After the leave-one-out cross-validation, the AUROC is 0.86.

We also compared the performance characteristics when using the SL-QPM–derived nuclear nanomorphology marker
alone, pathology alone, and combining pathology with the nuclear nanomorphology marker. Since the clinical goal is to detect malignant strictures with few false positives, we maximized the specificity to be 100% by choosing a cutoff value of $<OPD>$ to be 0.1548 µm to evaluate the performance characteristics of the SL-QPM. With this cutoff value, the sensitivity of SL-QPM alone is 73.1% at 100% specificity, while pathology alone gives 65.4% sensitivity at the same specificity. When combining SL-QPM with pathology, the sensitivity is significantly improved to 88.5% at 100% specificity. Of the nine false-negative cases based on pathology diagnosis alone, SL-QPM correctly identified six malignant strictures where malignancy was found during follow-up.

### Discussion

The diagnosis of malignancy on bile duct biopsy specimens obtained from patients with indeterminate strictures can often be difficult due to the small tissue size, superimposed inflammation, and the inherent difficulty of diagnosing malignancy. The reported sensitivity of conventional pathology evaluation of bile duct biopsy specimens ranges between 40% and 75%.

In this proof-of-concept study, we apply a novel optical microscopy system, SL-QPM, to analyze the epithelial nuclei on standard histology slides of bile duct biopsy specimens and demonstrate that SL-QPM–derived nuclear nanomorphology markers from bile duct biopsy specimens detect the presence of pancreaticobiliary malignancy, even from histologically normal-appearing (uninvolved) cells. The nuclear nanomorphology marker distinguishes benign from malignant strictures from histologically normal-appearing cells with a high level of statistical significance and discriminatory accuracy. Since this technique is directly using the standard histology slides without any modification, it is an objective, simple, low-cost test and has a great potential to be used as an adjunct diagnostic tool to improve the detection of malignancy in patients with an inconclusive pathologic diagnosis on bile duct biopsy specimens.

The presence of cancer-like signatures in histologically normal cells away from the primary tumor is a well-documented phenomenon in many tumor types, including pancreaticobiliary cancer, known as field effect, field defect, or field cancerization. Our previous studies have shown that the nuclear nanomorphology markers detect the presence of malignancy from histologically normal cells in multiple tumor types, such as colorectal, breast, and esophagus. This study further supports that nuclear nanomorphology markers also detect the presence of pancreaticobiliary cancer from histologically normal cells of bile duct biopsy specimens, suggesting that the nuclear nanomorphology markers could potentially serve as a common marker for the detection of field cancerization.

Although the exact biological mechanisms responsible for the altered nanomorphology characteristics in the cell nuclei are not known, the nuclear nanomorphology marker is the structural manifestation of complex molecular events detectable at the nanoscale. Several genetic mutations have been reported to detect the field effect in pancreaticobiliary cancer. For example, p53 mutations have been found in normal-appearing biliary tract mucosa adjacent to a malignancy. Such molecular events may lead to the changes in nuclear density and spatial arrangement of chromatin, which may be partially responsible for the alterations in nanomorphology markers.

Our suggested potential clinical use of this technique is to serve as a second-line diagnostic tool in patients who receive either an inconclusive pathologic diagnosis or a benign diagnosis but want to further eliminate the possibility of malignancy. While these results are encouraging, with demonstrated promise in detecting malignancy from histologically normal epithelial nuclei of bile duct biopsy specimens, there are a number of limitations in this study. First, this is a retrospective study, and the patient samples selected were not collected consecutively. Second, the sample size is relatively small, but the effect size was sufficient to achieve statistical significance. Furthermore, the use of a single marker (ie, nuclear $<OPD>$) in our prediction model mitigated the possibility of overfitting. Given our limited sample size, the diagnostic utility of this technique must be further validated in a larger independent patient population in a prospective manner.
This study shows that the SL-QPM–based analysis of a nuclear nanomorphology marker can detect the presence of pancreaticobiliary malignancy from bile duct biopsy specimens with a high level of accuracy. This technique could be used to improve the diagnosis of malignancy in cases for which conventional histology cannot make a definitive diagnosis. Since SL-QPM can be directly applied to standard histology slides without any modification, it has potential to be rapidly integrated into clinical practice at a low cost. Ultimately, a large multicenter prospective study would need to determine whether our proposed strategy can improve the detection of pancreaticobiliary malignancy from bile duct biopsy specimens in patients with a negative or an inconclusive pathologic diagnosis.

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