Early Detection of Lung Cancer with Meso Tetra (4-Carboxyphenyl) Porphyrin-Labeled Sputum

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Introduction: Early detection of lung cancer in high-risk individuals reduces mortality. Low-dose spiral computed tomography (LDCT) is the current standard but suffers from an exceedingly high false-positive rate (>96%) leading to unnecessary and potentially dangerous procedures. We, therefore, set out to develop a simple, noninvasive, and quantitative assay to detect lung cancer.

Methods: This proof-of-concept study evaluated the sensitivity/specificity of the CyPath Early Lung Cancer Detection Assay to correctly classify LDCT-confirmed cohorts of high-risk control (n = 102) and cancer (n = 26) subjects. Fluorescence intensity parameters of red fluorescent cells (RFCs) from tetra (4-carboxyphenyl) porphyrin (TCPP)-labeled lung sputum samples and subjects' baseline characteristics were assessed for their predictive power by multivariable logistic regression. A receiver operating characteristic curve was constructed to evaluate the sensitivity/specificity of the CyPath assay.

Results: RFCs were detectable in cancer subjects more often than in high-risk ones ($p = 0.015$), and their characteristics differed between cohorts. Two independent predictors of cancer were the mean of RFC average fluorescence intensity/area per subject ($p < 0.001$) and years smoked ($p = 0.003$). The CyPath-based classifier had an overall accuracy of 81% in the test population; false-positive rate of 40% and negative predictive value of 83%.

Conclusions: The tetra (4-carboxyphenyl) porphyrin -based CyPath assay correctly classified study participants into cancer or high-risk cohorts with considerable accuracy. Optimizing sputum collection, sample reading, and refining the classifier should improve sensitivity and specificity. The CyPath assay thus has the potential to complement LDCT screening or serve as a stand-alone approach for early lung cancer detection.

Key Words: Sputum cytology, Porphyrin, Early detection, Lung cancer.

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Individuals diagnosed with late-stage lung cancer have a 5-year overall survival rate of less than 17%.[1] Applying an actuarial model to the Surveillance, Epidemiology and End Results data, Goldberg et al.[2] estimated that early detection of lung cancer had the potential to save more than 70,000 lives per year.

The National Lung Screening Trial demonstrated that low-dose spiral computed tomography (LDCT) improved lung cancer outcome relative to conventional radiography.[3,4] LDCT is the standard procedure for early lung cancer detection[5] but suffers from a greater than 96% false-positive rate.[3,4] This means that for every three cancer patients correctly identified, 97 people are incorrectly scored as having lung cancer. Many of these people will undergo unnecessary follow-up procedures that carry their own morbidity and mortality. Thus, there is a dire need for approaches to complement or improve upon LDCT for early lung cancer detection.

An ideal early-screening method should be quantitative, noninvasive, highly sensitive and specific, and inexpensive enough to screen large at-risk populations. Obtaining and staining exfoliated material from sputum is relatively easy and inexpensive. Evidence also suggests that lung cancer detection across all stages, histological types, tumor sizes, and locations in sputum samples is possible[6]; however, microscopic observation of sputum samples without enhancement techniques is labor-intensive and yielding average sensitivities below 40%.[6] A recent multiparameter cytometric analysis of Feulgen-thionin–stained sputum reported a sensitivity of 78% at a specificity level of 50%.[7] Despite these advances, there is still a need for better screening methods to reduce morbidity and mortality.

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Lipophilic and amphiphilic porphyrins have been known since the late 1940s to exhibit high affinity for neoplastic tissue.[8] Cancer cell membranes have altered lipid membrane compositions and metabolism compared with noncancerous cells.[9–14] For example, most cancer cells show an increased uptake of low-density lipoprotein. Low-density lipoprotein's capability to bind porphyrin molecules[13,15,16] may explain in part the selective uptake of most porphyrins by cancer cells.[13] Certain porphyrin compounds produce a characteristic fluorescence that can be used for cancer detection.[17,18] The compound tetra (4-carboxyphenyl) porphyrin (TCPP) is particularly well suited for lung cancer detection because it contains carboxyl groups that become electrostatically neutral in the slightly acidic sputum-labeling environment, significantly improving its uptake into lipophilic cell membranes.[13,19,20] We have exploited TCPP's preferential binding and distinct fluorescent signature to develop an early lung cancer detection assay (CyPath). We describe herein the results of a proof-of-concept study conducted to determine the clinical sensitivity and specificity of this assay.

MATERIALS AND METHODS

Clinical Trial

Two minimal risk studies were registered with Clinical trials.gov (http://clinicaltrials.gov/ct2/show/NCT00894127 and http://clinicaltrials.gov/ct2/show/NCT02388074), reviewed and approved by the Quorum Institutional Review Board (Seattle, WA), and conducted according to ethical principles of the Declaration of Helsinki (v 1996) and Good Clinical Practice guidelines.

Subjects

The goal of the proof-of-concept study (NCT00894127) is to supplement LDCT for the diagnosis of lung cancer. We focused on determining the discriminative power of our assay between two highly comparable patient groups, and excluded healthy volunteers. The two groups included patients with confirmed lung cancer and people at high risk for developing lung cancer, but who were determined by a radiologist based on one or multiple LDCT scans to be cancer-free at the time of study conclusion.

Subjects greater than or equal to 18 years were recruited from military veterans who responded to public advertisements and cancer patients being seen at the investigative sites (Helen F. Graham Cancer Center Christiana Care Health System, Newark, DE and Waterbury Pulmonary Associates, Waterbury, CT) from December 2009 to February 2011. Individuals who agreed to have their medical information released as indicated and who signed informed consent were screened and—if they met eligibility criteria—enrolled in one of the two distinct study cohorts. The first group included individuals at high risk for developing lung cancer but presumptively cancer free; the second included individuals diagnosed with lung cancer (biopsy-proven stages I–IV primary or recurrent disease in the lung parenchyma).

To be eligible in the high-risk cohort, subjects had to be U.S. military veterans and heavy smokers (defined as ≥20 pack years). Inclusion of veterans in the high-risk group was stipulated by the State of New Mexico as a condition of study funding. For the second group, newly diagnosed lung cancer patients (not necessarily veterans) were treatment naive; those with recurrent tumors at least 1 year post therapy. Individuals with other primary cancers that had not metastasized to lung were eligible, but those with intrapulmonary metastases were not, nor were those with lung cancer that had metastasized to sites outside of the pleural cavity. Other exclusion criteria (applicable to both cohorts) were the presence of severe obstructive lung disease (forced expiratory volume/second <30% of predicted), uncontrolled asthma (defined as daily wheezing or hospitalization/emergency room visit within last year), angina with minimal exertion, dependence on supplemental oxygen, resting or room air oxygen saturation of 92% or 84%, pregnancy, or working in the mining industry.

Though not the target population for our assay, we also recruited a cohort of healthy participants (NCT0238807) to determine whether red fluorescent cells (RFCs) might usually occur even in the absence of disease or of high-risk factors. As anticipated, obtaining adequate deep lung sputum samples from this group was difficult as healthy lungs produce little usable material. We were nevertheless able to evaluate 5 out of 27 participants. For all other details on this trial, see Supplementary material (Supplemental Digital Content 1, http://links.lww.com/JTO/A859).

Study Design

All subjects participating in NCT00894127 underwent initial LDCT chest scans according to International Early Lung Cancer Action Program protocol.[21] In the high-risk cohort, LDCT scans were conducted at the investigational site (Radiology Associates of Albuquerque, Albuquerque, NM) and helped identify subjects who were potentially ineligible because of suspected lung cancers (i.e., presence of pulmonary nodules on LDCT that did not resolve before study closure). For the cancer cohort, LDCT was conducted at the investigational sites (Helen F. Graham Cancer Center and Waterbury Pulmonary Associates) and served to confirm the subject's lung cancer diagnosis and identify the location/extent of their disease.

Our initial intent was to include Papanicolaou (PAP) staining as a base of comparison to CyPath. Early evaluation, however, of the readings from the PAP-stained slides revealed that PAP could detect cancer cells in only 18.75% of the cancer cohort subjects (see Supplementary Tables A and B, Supplemental Digital Content 2, http://links.lww.com/JTO/A860, which show the results of the PAP-stained sputum slides for each cohort). With such poor sensitivity to detect true positives, it was decided to eliminate the CyPath comparison with PAP analysis from the study. PAP staining was thereafter only used to confirm the presence of macrophages, which signaled sample adequacy.

Laboratory technicians scoring CyPath-labeled samples, cytotechnologists scoring PAP specimens, and the radiologists reading the LDCT scans were blinded to the subjects' identity and cohort membership. CyPath, PAP, and LDCT results were not shared between specimen readers and radiologists.

Participants were not systematically informed about PAP or CyPath findings and no clinical recommendation or protocol-specific follow-up was based on PAP or CyPath results; however, subjects in the high-risk cohort who displayed at least one nodule on the initial LDCT scan were contacted, advised to have follow-up LDCT scans at the Radiology Associates of Albuquerque investigational site in accordance with International Early Lung Cancer Action Program recommendations[21] and were followed by telephone contact for at least 18 months. Follow-up LDCT scan results were provided to a radiologist who determined whether the subject's pulmonary nodules had resolved or stabilized and therefore could be considered cancer free and included in the high-risk group.

Sample Collection

All subjects participating in NCT00894127 were trained by the investigative site respiratory therapist/nurse on the first sample collection, using either the Lung Flute (Medical Acoustics; Buffalo, NY) or Acapella (Smiths Medical, St. Paul, MN). Both are FDA-approved, hand-held devices that help to thin and mobilize mucus secretions from within the lung. Medications that could impede sputum production were stopped within 2 days before sample collection. Subjects were instructed to use the device in accordance with manufacturer's instructions and expel the sputum sample into a numbered sterile collection cup. Individuals repeated this procedure at home to collect the second and third sputum samples. Those who could not produce a sample in the morning were asked to repeat the attempt one to two times that day or the next day. Subjects were instructed to store their specimen cup in a cool dark place or in a refrigerator and to return it to the site of initial collection within 3 days after collection was complete.

Of note, the majority of the sputum samples in this trial were collected with the Lung Flute. The Acapella was introduced as an alternative to the Lung Flute when 14 high-risk subjects were unable to produce satisfactory samples. When asked to repeat collection using the Acapella, 12 out of this same group of 14 subjects produced a sample of good quality (Supplementary Table 1, Supplemental Digital Content 3, http://links.lww.com/JTO/A861, which shows the results of the Lung Flute versus Acapella comparison).

Sample Processing

The specimen cup was delivered to the laboratory where each subject's specimen was transferred on the day of delivery to vials containing a buffered CytoLyt fixative solution (Hologic, Bedford, MA), pH 7 and stored at 2 to 8°C until processing. Specimens were centrifuged, the supernatant decanted, and the pellet resuspended in PreserveCyt solution (Hologic), pH 5.5. Thirteen slides per specimen, each with approximately 50,000 cells, were prepared with a ThinPrep machine (Hologic). Of these, 12 slides were immersed in the CyPath labeling solution and processed further for scoring (see below). One slide was shipped to Denver Veterans Administration Medical Center for PAP staining to evaluate for the presence of bronchial macrophages. Any unused pellet specimen was stored frozen at −20°C and discarded after study close.

PAP Staining

One slide from each subject was prepared on the ThinPrep platform (Hologic), and PAP staining was performed. This slide was evaluated by a cytopathologist. Samples were deemed adequate if they showed minimal mucous contamination, consisted of a monolayer of cells, and contained at least five free alveolar macrophages (FAMs) as a confirmation that the sample came indeed from within the lung; FAMs play an important defense role deep inside the lung, i.e., in the distal alveolar ducts and alveoli.[22] Individuals with inadequate samples were asked once to repeat the sputum collection procedure.

Labeling and Scoring

Labeling solution for the CyPath assay was prepared by dissolving TCPP (Frontier Scientific, Inc., Logan, UT) in isopropyl alcohol, to which deionized water, sodium bicarbonate (for pH adjustment), and 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer solution, pH 6.1, were added. For CyPath processing, specimen slides were first immersed in MES buffer, blotted dry, and then immersed in CyPath-labeling solution, blotted and rinsed again in MES before air drying and fixing with Cytoseal (Richard-Allan Scientific Cytology Reagents, Kalamazoo, MI).

Two laboratory technicians viewed the CyPath-labeled slides using an Olympus BX40 fluorescent microscope (Olympus Scientific Solutions Americas Corp., Waltham, MA) with a mercury vapor lamp filtered for 477.5 (±17.5) nm as the excitation source and an Olympus U-MWIB3 fluorescent filter cube. A standardized viewing protocol was followed. First, slides were screened for the presence of TCPP-labeled RFCs under 10 times magnification. RFCs were defined as circular/oval in shape with a defined perimeter and rounded edges, diameter greater than or equal to 8 μM, and fluorescing brightly red. Second, each RFC was evaluated under 20 times magnification for their characteristic spectral signature using CRi Nuance image analysis software (Cambridge Research and Instrumentation, Woburn, MA).

To estimate the background fluorescence intensity (FI) and size distributions of all cells (not just RFCs), one slide per subject was selected at random, and all cells within the field of view were measured as above at a series of predetermined coordinates until data for 100 cells had been captured. In addition to this background control, we also measured FI in sputum samples obtained from healthy volunteers (see Supplementary material, Supplemental Digital Content 3, http://links.lww.com/JTO/A861, which describes the results of the clinical trial for sputum analyses of healthy volunteers) showing that healthy sputum does not contain RFCs and that the background is very low compared with that of high-risk and cancer patients.

Statistical Analysis

In addition to RFC numbers per subject, quantitative measurements of 12 imaging parameters were collected on each RFC (collectively referred to hereafter as CyPath parameters). Fisher's exact test was used to compare the number of samples in each cohort that had at least one RFC. To construct

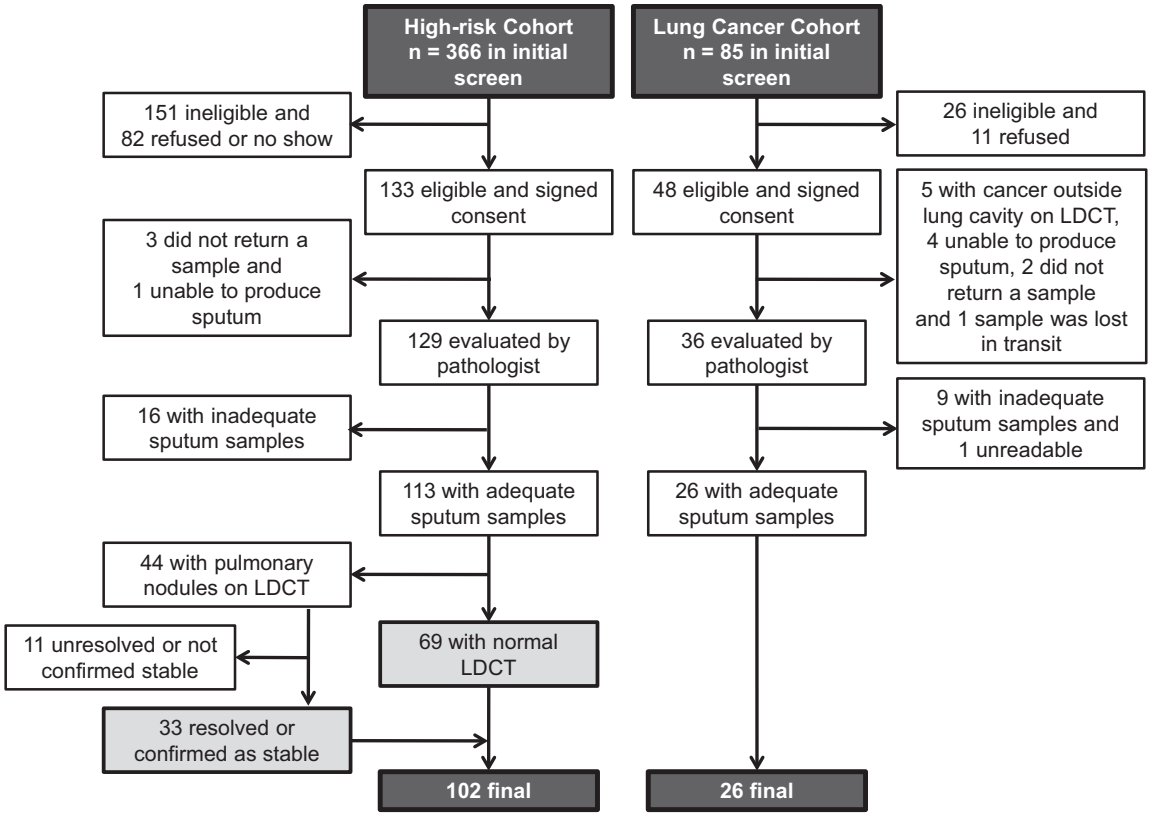

FIGURE 1. Subject enrollment and participation.

the classifier, a forward stepwise multivariable logistic regression analysis was performed using the Statistical Package for the Social Sciences software from IBM (Armonk, NY) to determine if any patient baseline characteristic(s) or CyPath parameter(s) were significant independent predictors of a subject's cohort membership. The regression coefficients of variables had to be significantly different from 0 ($p < 0.05$ initially and ≤ 0.10 after adjustment for other variables at any stage) to be retained in the model. The final fitted logistic model was used to estimate the probability of having cancer for each subject. Different cutoff values were used to convert the estimated probability into a binary cancer/no cancer classification. For each cutoff value, the associated sensitivity and specificity for the prediction, compared with the known cancer status of each subject, was determined and the results are plotted as a receiver operating characteristic (ROC) curve. A nonparametric rank test was used to compare the area under the ROC (AU-ROC) curve to an area of 0.5 achievable by chance alone.

RESULTS

Study Subjects and Baseline Characteristics

For the high-risk cohort (Fig. 1), a total of 366 veterans were informed about the study and underwent a screening interview. Of these, 233 were either ineligible, did not return after expressing initial interest, or refused to sign consent, and 133 were enrolled. Of these, 20 were unable to provide an adequate sputum sample. The initial LDCT scan was normal in 69 participants, but 44 had detectable pulmonary nodules. Of the 44 subjects with nodules, 33 either resolved or did not progress during follow-up. The remaining 11 subjects were excluded from the study as they could either not be contacted or the investigators could not confirm that their nodules had resolved by study closure. The high-risk cohort thus comprised 102 subjects (69 nodule-free subjects plus 33 with resolved or stable pulmonary nodules).

For the cancer cohort, a total of 85 lung cancer patients were informed about the study; 37 were either ineligible or refused to consent. Forty-eight agreed to participate and were enrolled (Fig. 1). The initial LDCT scan revealed that five of these cancer patients had lung cancer that had spread outside the pleural cavity and were therefore excluded per protocol. Seventeen subjects were unable to produce an adequate sputum sample. The remaining 26 participants comprised the final cancer cohort.

Table 1 shows baseline characteristics of the two cohorts. A higher proportion of male than female subjects were enrolled in each cohort, more so in the high-risk cohort, reflective of the gender composition among veterans. Consequently, gender was excluded from consideration as a predictive model parameter. The cancer cohort had a longer smoking history ($p = 0.010$).

Predictive Parameters

Cells identified as RFCs were distinct from surrounding background cells (Fig. 2) visually (*A*) and by their photon

TABLE 1. Demographics and Baseline Characteristics of Study Participants

	High Risk Cohort, N = 102	Lung Cancer Cohort,[a] N = 26	*P* Value
Males, n (%)	95 (93.1)	16 (61.5)	<0.001[b]
Females, n (%)	7 (6.9)	10 (38.5)	
Age, mean years ± SD (range)	57.2 ± 11.6 (28–90)	61.5 ± 9.3 (42–76)	0.081[c]
Smoking history			
Mean pack years ± SD (range)	49.4 ± 26.1 (18–51)	52.2 ± 38.2 (0–185)	0.667[c]
Mean years smoked ± SD (range)	33.0 ± 10.4 (12–60)	39.5 ± 14.7 (0–60)	0.010[c]
Mean years since stopped (range)	6.0 ± 10.2 (0–55)	3.6 ± 7.0 (0–29)[d]	0.186[c]

[a]The high-risk cohort excluded nonsmokers and the cancer cohort did not. Inclusion into the cancer cohort required that a participant had been diagnosed with lung cancer and that treatment had not started. Two of the cancer cohort subjects had never smoked.
[b]Fisher's exact test (two-sided).
[c]Student's *t* test (two-sided).
[d]n = 24 because two cancer cases never smoked.

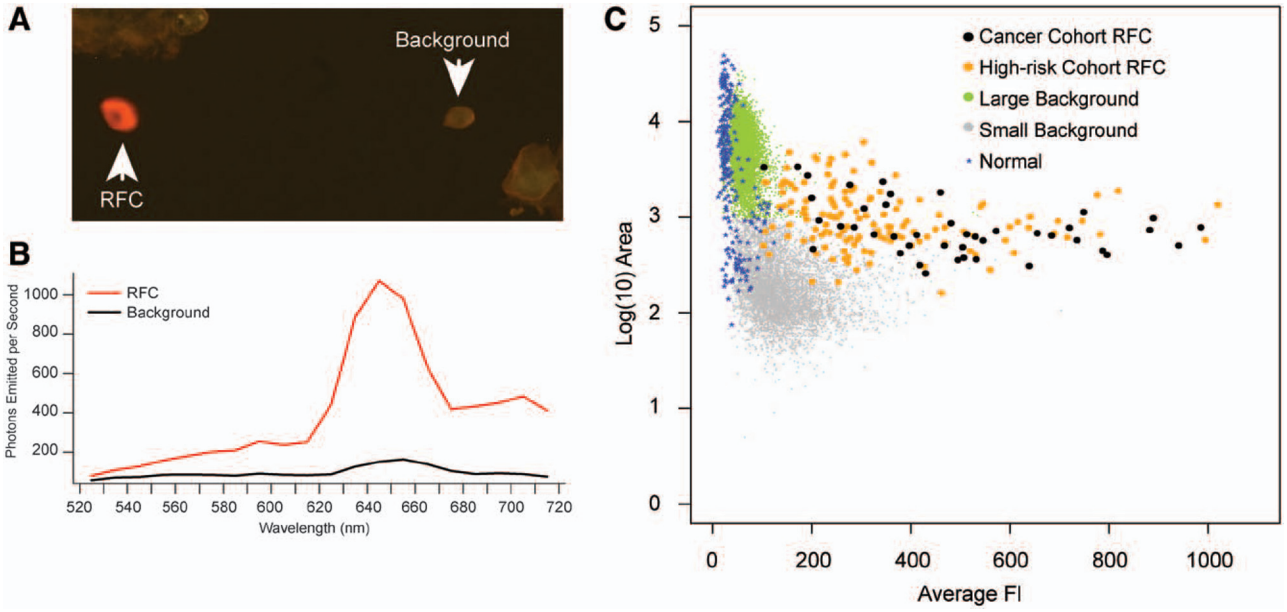

FIGURE 2. Characteristics of CyPath-stained cells. *A*, The intense red color of red fluorescent cells (RFCs) indicates uptake of tetra (4-carboxyphenyl) porphyrin (TCPP) and demonstrates a characteristic fluorescence when viewed after exposure to blue light (of a mercury vapor light source) through an Olympus U-MWIB3 filter. This is in contrast to surrounding background cells (*A*, background) which do not take up TCPP and appear dull when viewed under these conditions. *B*, Spectral emission profiles for the cells identified by arrowheads in *A*. *C*, Average fluorescence intensity (FI) versus $\log_{10}$ area of cells chosen at random (green, grey, blue) and of RFCs from high-risk (orange) and cancer (black) cohorts. The distinction between large and small was based on the *k*-means ($k = 2$) partitioning of the $\log_{10}$ area of background cells.

emission profiles (*B*). No RFCs were detected in slides from healthy participants. Overall, a significantly higher proportion of subjects with cancer (20 of 26, 76.9%) had greater than or equal to one RFC than the high-risk group (51 of 102, 50%; $p = 0.015$). Nevertheless, RFCs could be detected in both groups, so that the presence of RFCs in and of itself was not a sufficient discriminator of class membership. We, therefore, undertook to identify key characteristics of the subjects and of the RFCs from each of the two test cohort.

The CyPath parameters (Table 2) included several direct FI values, cell size measurements, measures of FI scatter (SD), and derived ratios of FI/area. Between cohorts, differences were statistically significant for two CyPath parameters and one approached significance. These were the mean of RFC per subject of average FI (FI_{avg_mean}, $p = 0.017$), mean per subject of maximum FI (FI_{max_mean}, $p = 0.032$), and the mean ratio of FI_{avg}/area ($[FI_{avg}/area]_{mean}$, $p = 0.051$).

All CyPath parameters listed in Table 2 plus subject age, pack years of smoking, and number of years smoked were evaluated as candidate predictors. The aim of this approach was to identify features whose inclusion in the predictive model significantly improved the model's goodness-of-fit to the data. Of the demographic and baseline characteristics assessed, the number of years smoked was a significant predictor of cohort membership ($p = 0.003$). Of the CyPath parameters, the $(FI_{avg}/area)_{mean}$ was a significant predictor

TABLE 2. CyPath Parameter Values for Subjects with Greater than or Equal to One Red Fluorescent Cell (RFC)

	High-Risk Cohort (n = 51)	Cancer Cohort (n = 20)	*P* Value
No. of RFCs	2.6 ± 3.0 (1–19)	2.2 ± 2.0 (1–9)	0.537
Mean RFC FI_{avg} (FI_{avg_mean})	346.5 ± 154.9 (106–898)	457.3 ± 209.1 (104–889)	0.017
Mean RFC FI_{max} (FI_{max_mean})	410.5 ± 200.3 (106–1019)	533.4 ± 266.1 (104–985)	0.072
Maximum FI_{avg} (FI_{avg_max})	685.7 ± 306.9 (298–1985)	871.9 ± 362.4 (264–1714)	0.032
Maximum FI_{max} (FI_{max_max})	815.3 ± 423.5 (298–2803)	988.2 ± 422.0 (264–1787)	0.126
Mean cell diameter (pixel diameters[a])	33.1 ± 10.5 (18–65)	33.0 ± 12.6 (18–61)	0.954
Maximum cell diameter (pixel diameters)	38.1 ± 14.7 (18–82)	37.3 ± 14.9 (18–63)	0.838
Mean cell area (pixels[b])	1150.3 ± 745.7 (304–3730)	1129.9 ± 899.6 (258–3336)	0.922
Maximum cell area (pixels)	1558.2 ± 1266.5 (304–6109)	1317.0 ± 952.5 (258–3368)	0.444
Mean FI_{SD} (FI_{SD_mean})	130.1 ± 73.4 (45–353)	165.9 ± 81.7 (31–353)	0.078
Mean maximum FI_{SD} (FI_{SD_max})	160.2 ± 96.0 (45–487)	191.4 ± 90.1 (31–355)	0.214
Mean ratio of FI_{avg}/area ($[FI_{avg}/area]_{mean}$)	0.478 ± 0.311 (0.05–1.40)	0.751 ± 0.562 (0.03–1.97)	0.051
Mean ratio of FI_{max}/area ($[FI_{max}/area]_{mean}$)	0.661 ± 0.530 (0.05–2.87)	0.910 ± 0.690 (0.03–2.07)	0.157

FI measurements are the number of photons emitted per pixel per half-second.
Parameters significantly (or borderline) different between groups are shaded grey.
Mean values are shown ± standard deviation with ranges shown beneath in parentheses.
[a]Pixel diameter = 0.492 μm.
[b]Pixel = 0.242 μm^2.
FI_{avg}, average fluorescence intensity; FI_{max}, maximum fluorescence intensity; FI_{SD}, standard deviation fluorescence intensity.

TABLE 3. Multivariable Logistic Regression Model Predictors of Cohort Membership

	B	SE	Wald	df	*P* Value	Odds Ratio (OR)	95% CI for OR	
							Lower	Upper
Mean (FI_{avg}/area)	2.164	0.574	14.220	1	<0.001	8.707	2.827	26.814
No. of years smoked	0.066	0.022	8.778	1	0.003	1.068	1.022	1.115
Constant	−4.576	0.979	21.824	1	<0.001	0.010		

B, intercept; SE, standard error around B; Wald, *P* value of the Wald chi-square test; df, degree of freedom; CI, confidence interval.

($p < 0.001$). The other factors assessed were not significant and therefore not included in the model. Figure 2*C* shows the distribution of average FI versus $\log_{10}$ area of RFCs from the two test groups and of randomly selected cells from their slides and from healthy participant slides as a background control. RFCs from both cohorts were of an intermediate size. There was a tendency for the RFCs from the high-risk cohort to be dimmer as a group, consistent with $(FI_{avg}/area)_{mean}$ as a predictor of group membership.

After adjusting for $(FI_{avg}/area)_{mean}$ and pack years, all remaining variables had *p* values greater than 0.10 and were, therefore, excluded from the final model. Along with the other model metrics, Table 3 shows that the odds ratio (OR) for the CyPath parameter, $(FI_{avg}/area)_{mean}$, was 8.707, whereas the OR of years smoked was 1.068, indicating a greater impact of the CyPath variable on predicting the likelihood of a subject having cancer.

Sensitivity and Specificity

Absence of RFCs in the CyPath-stained slides of 6 out of 26 subjects with cancer suggests a potential upper bound on sensitivity for cancer detection of 77.9% when 12 slides (or ~600,000 cells) are scanned; resulting in a corresponding specificity of 65.7%. The predictive capabilities of the CyPath assay classifier for cases with RFCs detected were estimated from the final fitted model with $(FI_{avg}/area)_{mean}$ and number of years smoked as its variables. The ROC curve in Figure 3 shows the response in true-positive (sensitivity) and false-positive (1-specificity) rates as we varied the fitted model probability estimate cutoff. The AU-ROC curve was 0.77 ± 0.51 (standard error under the nonparametric assumption; 95% confidence interval: 0.67–0.87), significantly greater than the AU-ROC of 0.50 theoretically achievable by random assignment of subjects to cancer/control groups ($p < 0.001$), indicating that the CyPath assay can distinguish between true positives and true negatives significantly better than chance alone.

The standard cutoff probability of 0.5 was used to estimate overall predictive accuracy. The fitted model in this case correctly classified 98 out of 102 (96.1%) high-risk participants and 6 out of 26 (23.1%) cancer subjects, for an overall accuracy of 81.3%, a false discovery rate of 40%, and negative predictive value of 83%.

DISCUSSION

Screening for lung cancer using sputum is appealing because it is noninvasive, simple, requires no exposure to radiation, and is relatively inexpensive, although having the potential to detect very early disease.[7,23] Overall, 88% of the high-risk group was able to provide a sample with FAMs

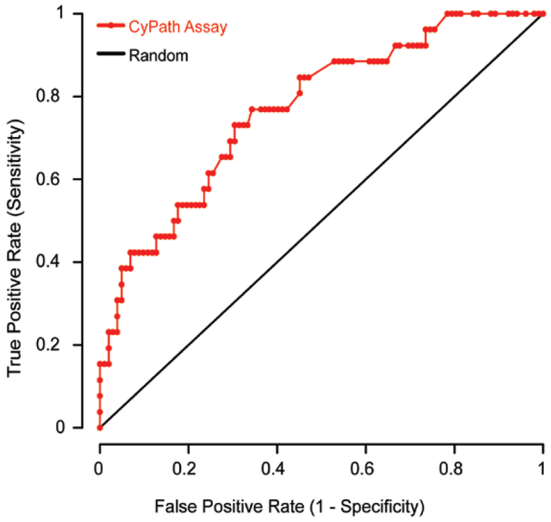

FIGURE 3. Receiver operating characteristic curve for the CyPath assay classifier. The accuracy of classifying each participant as cancer/no cancer was tabulated as the probability cutoff for the predictive model was varied. The red curve shows the changes in specificity and sensitivity for the CyPath assay at the different probability thresholds. The black line indicates the expected result if subjects were assigned randomly.

associated with deep lung expectoration. Use of the Acapella device, in particular, seemed to result in a better sputum production than with the Lung Flute. In both cases, collection was accomplished fairly quickly with little difficulty and no adverse consequences for the subject.

The fluorescence emission characteristics of CyPath-labeled cells were quantifiable and distinguishable from background, making the identification of RFCs straightforward. Within the positive control cancer cohort, 23% of participants had no RFCs detected in the 12 slides (~600,000 cells) evaluated. If RFCs are distributed randomly according to a Poisson distribution in the cancer samples, our results suggest a frequency of roughly 1.5 RFC per 12 slides (20 of 26 with at least one RFC). We would, therefore, expect that doubling the number of cells examined, for example, could increase RFC detection to 95%.

Age and pack years of smoking are clearly risk factors for developing lung cancer, but they are less accurate than risk prediction models in identifying people who will develop lung cancer and die from the disease.[24] The aim of this proof-of-concept study was to determine whether CyPath labeling could be usefully incorporated into a predictive model. We found that the number of years smoked was a predictor of being in the cancer cohort though the associated OR was a modest 1.1. Importantly, one of the RFC parameters, mean FI_{avg}/Area, also had a positive predictive value (PPV) with an OR of 8.7. When combined in a classifier, these two variables allowed us to correctly assign subjects to the cancer or high-risk cohorts with an accuracy of 81% and PPV of 60% on this data set. Our focus in this study was to determine the discriminatory power of our assay between two highly similar patient groups. Our results indicate that further optimization of predictive model parameters and of sputum harvesting and processing would likely improve our classification accuracy for these populations.

We have deliberately excluded patients with noncancerous pulmonary diseases from consideration to maintain the focus of this analyses, but our findings now make a compelling case for exploring further applications of CyPath labeling to these diseases. Others have reported that porphyrins are preferentially taken up by cancer cells,[8] and this study shows that features of RFCs are positive discriminators for the presence of cancer. Moreover, we observed that the $(FI_{avg}/area)_{mean}$ of RFCs in cancer patients diagnosed with adenocarcinoma was higher than that in patients with squamous carcinoma (data not shown). Together, these observations suggest that RFCs are related to lung cancer. The true nature of RFCs remains unclear because they were also found in sputum samples from the high-risk group, who during the period of the trial were not diagnosed with cancer. The follow-up of patients in our study was relatively short. It is thus possible that in some of these individuals the RFCs present early signs of cancer, before it became obvious by LDCT. Importantly, our data show that the $(FI_{avg}/area)_{mean}$ of the RFCs detected in the sputum of the high-risk cohort is lower than in the cancer cohort. This may present a true biological difference, e.g., a different cell type, and follow-up studies will address this issue. Moreover, future studies will also be aimed at identifying a cut-off value of the $(FI_{avg}/area)_{mean}$ above which a cancer diagnosis is more likely, thus further improving our classification accuracy.

In conclusion, the CyPath assay has the potential to satisfy the requirements of an early lung cancer screening methodology: the starting material is readily acquired, the staining protocol is straightforward and scalable, evaluation of TCPP staining is both quantitative and amenable to high-throughput processing, and the classifier based on smoking history and TCPP fluorescence characteristics is already reasonably accurate and predictive. Our proof-of-concept results are particularly encouraging when compared with the corresponding values for LDCT of 74% accuracy and 3.8% PPV.[4] Further development of this promising technology, followed by validation trials in lung cancer patients and control groups, therefore, seems both warranted and of potential future clinical value.

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