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EarlyCDT-Lung Overview

GeneNews has licensed EarlyCDT-lung from UK-based, Oncimmune®, for marketing, sale and distribution throughout the U.S. EarlyCDT-Lung is a sophisticated blood test that aids physicians in risk assessment and the early detection of lung cancer in high-risk patients. The EarlyCDT-Lung test is a blood test based on enzyme linked immunosorbent assay (ELISA) principles. It uses microtiter plates coated with a set of serial dilutions of recombinant antigens. Samples are judged to be positive for the presence of autoantibodies when they (i) show a dose response to the antigen titration series and (ii) have a signal above the cut-point for an assay to one or more of the antigens. Test cut-off values were selected using Monte Carlo search methods and then tested prospectively on independent datasets.

During tumorigenesis, normal cells produce a number of novel, aberrantly expressed or mutated proteins (autoantigens) which are recognized by the immune system as ‘non-self’ and elicit the production of antibodies against them (‘autoantibodies’). Autoantibodies arise in the early stages of lung cancer development, can also be present at later stages, and exist in sufficient quantity and size to be measurable in blood even when the tumor may be small and/or localized. Elevated levels of autoantibodies beyond a predetermined cutoff are indicative of disease, whereby the test indicates increased risk of lung cancer, necessitating a follow-up test(s) [e.g., Computed Tomography (CT)] in accordance with current guidelines for management of pulmonary nodules (e.g., the Fleischner guidelines).

Elevation of any one of the seven autoantibodies (p53, NY-ESO-1, CAGE, GBU4-5, MAGE-A4, SOX2, HuD) above a predetermined cut-off value suggests that a tumor may be present or will develop.3,4,5 If the autoantibody levels all lie below the cutoff this does not mean the patient is cancer-free; he or she is still at high risk because of associated risk factors, such as smoking, whereby monitoring via follow-up testing with EarlyCDT-Lung is recommended, along with adherence to current USPSTF lung cancer screening guidelines.

The key advantage of EarlyCDT-Lung is its ability to detect lung cancer earlier, and with higher sensitivity and specificity than low dose CT alone, which is the current standard diagnostic imaging test.
A Dual Approach for Lung Cancer Screening

While the performance metrics of EarlyCDT-Lung compares favorably with annual CT screening it is the combination of both tests that yields the most benefit. EarlyCDT-Lung offers a complementary approach to annual CT screening which is the standard in the USA for early lung cancer detection if the patient meets the high-risk criteria set by USPSTF. As a convenient blood test, EarlyCDT-Lung can be used when an individual is at increased risk, but does not meet the criteria for annual CT screening or, when individuals are unwilling or unable to undergo lifelong annual CT screening. In either case, a Moderate or High Level EarlyCDT-Lung result can be followed by suitable CT scans to confirm the presence of lung cancer.

EarlyCDT-lung, in the 12,000 patient National Health Service, Scotland study, has shown that it can detect lung cancer as much as 2 or more years before it may be seen via LDCT. A raised risk score, followed up aggressively for 2 years, demonstrated a sensitivity of 81%, while maintaining a specificity of 91%. This contrasts with the validation studies where, with only a six month follow up, sensitivity was 40%. The difference is in aggressive follow-up for a significant enough time to allow LDCT to see it. LDCT, while the standard for screening for lung cancer, typically will not differentiate well below a range of 2-4 mm. Also, there is an issue with differentiating between benign and malignant growths, with a false positive rate of 96%. A combination of EarlyCDT-lung and LDCT should deliver a superior result than LDCT alone.

The National Health Service ("NHS") Scotland-sponsored Early Cancer Detection Test – Lung Cancer Scotland ("ECLS") Study of 12,000 high-risk smokers demonstrated a cancer detection rate (sensitivity) of 81% for EarlyCDT®-Lung in these initial results. While the control arm in the study has not been formally assessed, the positivity rate was as expected with a specificity of 91%. The final data on the control arm will be collected at the end of the study.

The value of EarlyCDT-Lung lies in its ability to detect early stage disease (Stages I and II) and multiple histological subtypes, with an overall specificity of 93% (specificity of a High Risk result improves to 98%), positive predictive value of 1 in 10 (PPV improves to 1 in 5 for a High Risk result) and overall accuracy of 92% (97% for a High Risk result), assuming a lung cancer prevalence of 1.2%. 

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EarlyCDT-Lung Validation

A key ingredient to EarlyCDT-Lung's success is its extensive scientific and clinical validation studies. More than 120,000 patient samples were run and 12 million data points analyzed to validate the technical and clinical performance of EarlyCDT-Lung. Since then over 145,000 commercial tests have also been run in the US laboratory.

EarlyCDT-Lung is being used in the largest randomized trial for the early detection of lung cancer using biomarkers ever conducted; the National Health Service (NHS) Scotland ECLS study of 12,000 high-risk smokers.

To see a selection of our clinical publications please go to Scientific Papers. Murray et al. reported the laboratory validation data and performance characteristics for the serum autoantibody test panel consisting of six tumor associated antigens (EarlyCDT-Lung test). The authors specifically address the development of quality assurance in reagent preparation, analyte calibration and quality control (QC) protocols. Three separate groups of patients with newly diagnosed lung cancer and normal controls were identified. Patients with lung cancer were as far as possible individually matched to a control individual with no previous history of malignant disease (based on age, sex and smoking history). Blood samples were taken from all participants, and were obtained after diagnosis but before the start of any treatment in patients with lung cancer. ¹

In the initial validation studies the results confirm the reproducibility of the EarlyCDT-Lung test for identifying almost 40% of primary lung cancers. In the recent NHS study with a two year follow-up confirms the ability to identify 80% of lung cancers.(NHS Study Protocol Data on File) The data also confirm the value of a panel of autoantibodies over a single autoantibody assay; when the same antigens are used, the overall sensitivity and specificity remains constant even in discrete populations. Finally, the stability of the results between protein batches allowed for the development of formal procedures for batch verification and release that ensure that all the required QC criteria are met and that an antigen batch is validated before it is released for use in the assay. This will ensure continuity of results across multiple antigen batches. ¹

Scientific Papers


Audit of the autoantibody test, EarlyCDT®-Lung, in 1600 patients: An evaluation of its performance in routine clinical practice


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Objectives: EarlyCDT®-Lung may enhance detection of early stage lung cancer by aiding physicians in assessing high-risk patients through measurement of biological markers (i.e., autoantibodies). The test’s performance characteristics in routine clinical practice were evaluated by auditing clinical outcomes of 1613 US patients deemed at high risk for lung cancer by their physician, who ordered the EarlyCDT-Lung test for their patient.

Methods: Clinical outcomes for all 1613 patients who provided HIPAA authorization are reported. Clinical data were collected from each patient’s treating physician. Pathology reports when available were reviewed for diagnostic classification. Staging was assessed on histology, otherwise on imaging.

Results: Six month follow-up for the positives/negatives was 99%/93%. Sixty-one patients (4%) were identified with lung cancer, 25 of whom tested positive by EarlyCDT-Lung (sensitivity = 41%). A positive EarlyCDT-Lung test on the current panel was associated with a 5.4-fold increase in lung cancer incidence versus a negative. Importantly, 57% (8/14) of non-small cell lung cancers detected as positive (where stage was known) were stage I or II.

Conclusions: EarlyCDT-Lung has been extensively tested and validated in case–control settings and has now been shown in this audit to perform in routine clinical practice as predicted. EarlyCDT-Lung may be a complementary tool to CT for detection of early lung cancer.

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1. Introduction

Lung cancer currently causes more deaths from cancer in the world than any other tumor type, and projections over the next 20 years indicate this is likely to continue unless substantial progress is made in areas such as screening, early detection, treatment and prevention. The National Lung Screening Trial (NLST) addressed the question of CT screening and early detection in a large randomized trial and reported a 20% reduction in lung cancer mortality [1]. This provided level 1 evidence and confirmation of previous non-randomized trials of CT screening [2–5] that reported more detection of early stage disease and prolonged survival.

The fact that we now know that screening and early detection saves lives from lung cancer is in many ways only the start of the process of developing a cost effective early detection program. A screening program based only upon CT as demonstrated by the NLST study has numerous problems, including a high number of benign nodules identified (i.e., false positives; e.g., 96.4% of the positive results in the NLST study were benign) [1,2,6,7], the lingering question of what to do after 3 annual screens, and the fact that only ∼30% of all lung cancer patients would meet the NLST entry criteria (i.e., 55–74 years of age, ≥30 pack-years smoking history, and if an ex-smoker, must have quit within the last 15 years) [1]. One recent publication from a single US center focused on patients presenting with early stage lung cancers and aimed to address the question of the percentage of patients with early stage lung cancer who fulfilled the NLST criteria. Based on 267 patients with early stage disease, less than half met the NLST high risk criteria. Since the majority of these patients were not considered high-risk by the NLST criteria, they would not be covered under current screening paradigms [8].

It therefore seems that a requirement for an effective early detection program would be a biological test that would increase...
the pre-test probability of lung cancer in a high risk population – the pre-test probability being based either on demographic factors (e.g., age and smoking history), imaging findings (e.g., lung nodules) or both. A biological test that is performed on a peripheral blood sample would have clear advantages, including patient compliance, convenience and cost savings. EarlyCDT-Lung is a blood test that measures autoantibodies to lung cancer-associated antigens. It was developed to aid physicians in the early detection of lung cancer in a high-risk population. EarlyCDT-Lung was introduced clinically in a limited manner; as part of the limited release of the test a clinical audit program was established for individuals who gave consent for follow-up in accordance with the HIPAA Privacy Rule. The primary purpose of the audit was to confirm that the characteristics of the test, as reported in the training and validation case-control studies, were reproducible in routine clinical practice. This manuscript reports clinical outcomes at 6 months following EarlyCDT-Lung for the first ~1600 patients whose physicians ordered the test and where the patient gave informed consent to be part of the audit program.

2. Patients and methods

2.1. Audit population

The first 1699 patients for whom US physicians ordered EarlyCDT®-Lung are described here. The tests were ordered by 810 unique physicians in 720 different practices throughout 48 US states. As this is an audit of clinical practice, we are reporting the physicians’ use of the test and not a prospective study in a population defined by inclusion and exclusion factors. Of these 1699 patients, 1613 (95%) signed a HIPAA authorization permitting the development of sub-groups in routine practice. The patient demographics of the overall audit population (n = 1613) and the 6AAB (n = 752) and 7AAB (n = 861) panel groups are shown in Table 1 along with the 5-year lung cancer risk for the groups tested on the 6AAB and 7AAB EarlyCDT-Lung panels. The overall percentage of individuals followed-up was 99% and 93%, respectively (Table 2); these data are also further broken down by the 6AAB and 7AAB groups (Table 2).

2.2. EarlyCDT-Lung assay

EarlyCDT®-Lung is a physician-ordered blood test that serves as a tool to aid in early detection of lung cancer in high-risk patients. The test is performed only in Oncimmune’s CLIA laboratory (De Soto, KS). The technology has been extensively validated and has been shown to be technically and clinically robust [9,11–13]. EarlyCDT-Lung detects the presence of AABs to a panel of lung cancer-associated antigens using a semi-automated indirect ELISA-based method. A test result was reported as positive if the antigen titeration series showed a dose response and any one or more AAB levels were elevated above the clinical cut-off.

Testing of all patient specimens by EarlyCDT-Lung was performed in Oncimmune’s CLIA laboratory, including the data handling and calculation of the test result, which was performed by the Oncimmune laboratory information management system (LIMS); final test results were generated and reported to individual physicians. All EarlyCDT-Lung tests were performed prospectively upon receiving the physician’s order, and the results were reported back to the physician without knowledge of the patient’s clinical outcome, which was subsequently obtained as part of this audit.

2.3. Audit plan

Demographic data were requested as part of the EarlyCDT-Lung test requisition form. These data were considered in the audit. Additionally, clinical follow-up data on patients who provided HIPAA authorization were collected from their treating physician. In patients with a positive EarlyCDT-Lung test, contact was made with physicians immediately following the reporting of the EarlyCDT-Lung result and maintained until the physician indicated that a diagnosis had been reached or a follow-up plan decided (i.e., anticipated timing of imaging, biopsy, surgery, etc.); this was usually within 2–3 months of the EarlyCDT-Lung test. Subsequent follow-up on patients with a positive EarlyCDT-Lung test was then structured around the physician-described follow-up plan. Information concerning whether a patient was diagnosed with cancer was requested from physicians for all individuals regardless of test result at 6 months after the test. This timeframe was chosen (i) because it was felt to represent a timeframe within which the immediate value of a positive test result could be assessed, (ii) it allowed time for all patients with a negative EarlyCDT-Lung test to present with lung cancer in order to reduce the chance of observer bias in preferentially following up individuals with a positive EarlyCDT-Lung test result. One patient with a positive test was diagnosed just outside the 6 month period: this patient has been included since they were being actively investigated during the six month period for a lesion identified on imaging as being suspicious of lung cancer. The overall percentage of individuals followed-up at six months in the positive and negative EarlyCDT-Lung groups was 99% and 93%, respectively (Table 2); these data are also further broken down by the 6AAB and 7AAB groups (Table 2).
This report, therefore, focuses on the initial presentation and outcomes of all patients within 6 months following testing by EarlyCDT-Lung. Wherever possible, histology/cytology reports were reviewed and considered for diagnostic classification; some patients did not have a tissue diagnosis but were diagnosed, for example, based on imaging reports. It was decided from the start of the audit that if a physician diagnosed a lung cancer, then only in circumstances where there was specific proof to the contrary, and this was confirmed by an external expert, would the diagnosis by the treating physician not be accepted; this rule was applied for all patients regardless of EarlyCDT-Lung result.

2.4. Statistical analyses

The EarlyCDT-Lung test performance is presented in terms of standard test characteristics, such as sensitivity (the percentage of true positives) and specificity (the percentage of true negatives). Positive predictive value (PPV; the probability of cancer given a positive test result) was also calculated. These analyses were performed using Microsoft Excel. Comparison of sensitivity and specificity of EarlyCDT-Lung for the 6AAB and 7AAB groups is also presented; these comparisons were made using chi-squared tests.

3. Results

Of the 1613 test results, there were 14 patients where the test result was declared ‘Invalid’ (by pre-determined criteria, as outlined in the laboratory’s standard operating procedures) on the report sent to the treating physician. There were 222 patients who tested positive (14%) and 1377 tested negative (86%) (Fig. 1). The percent positive for the 6AAB and 7AAB panels was 18% (n = 139) and 10% (n = 83), respectively.

Sixty-one patients (4%) were diagnosed with lung cancer within 6 months following EarlyCDT-Lung, 25 of whom tested positive by EarlyCDT-Lung (i.e., 25 true positives and 36 false negatives; sensitivity = 41%). There were 1341/1538 patients not diagnosed with lung cancer who tested negative (i.e., 1341 true negatives and 197 false positives; specificity = 87%). The correlation between the EarlyCDT-Lung result and clinical outcome in terms of diagnosis within six months after having taken the EarlyCDT-Lung test is shown in Fig. 1 and Table 3. Comparing performance of the two panels, the 7AAB panel showed highly statistically significant (p < 0.0001) improvements in specificity over the 6AAB panel with 91% specificity for the 7AAB panel (i.e., 742 true negatives and 70 false positives) and 83% specificity for the 6AAB panel (i.e., 599 true negatives and 127 false positives). The sensitivities of the 6AAB and 7AAB panels were not statistically different (p = 0.5): 46% (i.e., 12 true positives and 14 false negatives) versus 37% (i.e., 13 true positives and 22 false negatives), respectively. The improvement in PPV offered by the 7AAB panel was nearly 2× better than the previous 6AAB panel: 16% (1 in 6.4) for the 6AAB panel versus 9% (1 in 11.6) for the 6AAB panel (Table 3).

Of the 61 lung cancer cases diagnosed, 46 (75%) were non-small cell lung cancer (NSCLC), 4 (7%) were small cell lung cancer (SCLC), 1 (2%) was mixed NSCLC–SCLC, and type was unknown for 10 (16%) cases (Table 4). Of the 46 NSCLCs with a histologic diagnosis, 26 (57%) were early-stage (stage I or II), 16 (35%) were late-stage (stage III or IV) and 4 (9%) were stage unknown (Table 4). Importantly, 57% (8/14) of NSCLCs detected as positive by EarlyCDT-Lung (where stage was known) were early-stage. Stage was unknown for an additional 2 NSCLCs detected by EarlyCDT-Lung. Thirty-two NSCLCs were adenocarcinoma and 14 were squamous cell carcinoma. Only four cases of small cell lung cancer were diagnosed, which is too few to allow for further evaluation. Of the 10 patients with unknown type of lung cancer (Table 4), 9 were diagnosed clinically due to the patient’s condition being too fragile for biopsy
Breakdown of cancer type/sub-type and stage of disease for the 61 lung cancer cases.

<table>
<thead>
<tr>
<th>Lung cancer type/sub-type</th>
<th>Number</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>NSCLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>32 (52%)</td>
<td>13 (41%)</td>
</tr>
<tr>
<td>Squamous</td>
<td>14 (23%)</td>
<td>8 (57%)</td>
</tr>
<tr>
<td>Total (NSCLC)</td>
<td>46</td>
<td>21 (46%)</td>
</tr>
<tr>
<td>SCLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small cell lung cancer (SCLC)</td>
<td>4 (7%)</td>
<td>–</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed SCLC + NSCLC</td>
<td>1 (2%)</td>
<td>–</td>
</tr>
<tr>
<td>Unknown type*</td>
<td>10 (16%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Overall total</td>
<td>61</td>
<td>24 (39%)</td>
</tr>
</tbody>
</table>

* "Unknown Type" refers to those patients with a clinical diagnosis of lung cancer who were too fragile for biopsy, had an inconclusive biopsy, declined further testing or whose records were unavailable.

(n = 4), an inconclusive biopsy (n = 3) or the patient refused diagnostic procedures (n = 2), and in 1 case the information was not accessible due to the patient’s records being in storage.

4. Discussion

The performance characteristics of the EarlyCDT-Lung test in clinical practice, as demonstrated by this prospective audit, mirrors that of the extensive case–control training and validation studies previously reported [9,12–14]. This audit has confirmed that EarlyCDT-Lung detects all types of lung cancer, all stages of the disease, and performs in clinical practice with the same sensitivity and specificity measured in the case–control studies. This is, therefore, the first autoantibody test that detects early stage lung cancer as shown with prospective validation data on a large number of individuals from a routine clinical practice setting.

Furthermore, the previously reported change that was made to the panel in November 2010 (6AAB panel to 7AAB panel) [9] has proven in routine clinical practice to have reduced the number of false positives (i.e., increased specificity), while maintaining the same ability to detect lung cancers (i.e., sensitivity). This resulted in an increased PPV of EarlyCDT-Lung in routine clinical practice from 9% (1 in 11.6) with the 6AAB panel to 16% (1 in 6.4) with the 7AAB panel (Table 3). For patients with a negative EarlyCDT-Lung result on the current 7AAB panel, 22/764 (3%) were found to have a lung cancer (i.e., 1 in 34.7). Thus, a positive result on the current 7AAB EarlyCDT-Lung test panel represents, on average, a 5.4-fold increased incidence of lung cancer within 6 months.

According to the National Cancer Institute’s SEER statistics, 39% of lung cancers are adenocarcinoma, 21% are squamous cell, and 14% are SCLC [15]. With the exception of a slightly higher proportion of adenocarcinoma (52%) and lower proportion of SCLC (7%) in our group, our audit findings are in line with the SEER statistics’ breakdown by histological sub-type, confirming that the cohort presented here is representative of a high-risk (for lung cancer) population and is not heavily biased toward any particular type of lung cancer. These audit data also confirm the case–control validation results that EarlyCDT-Lung detects all sub-types of lung cancer.

EarlyCDT-Lung has been shown in case–control studies and now in this clinical audit to also detect early-stage lung cancer. In the group evaluated for this audit where stage was known, 57% (8/14) of NSCLCs detected by EarlyCDT-Lung were early-stage.

The results presented on the overall performance characteristics of the test (e.g., specificity and sensitivity) confirm that in routine clinical practice EarlyCDT-Lung performs as predicted from our previously reported large case–control studies. The audit results have highlighted the value of the test to physicians as an aid to detection of early lung cancer.

Until recently, there were no significant biological markers related to the individual or the lung cancer that could be measured as a blood test and used in clinical practice. EarlyCDT-Lung measures AABs to lung cancer-associated antigens; it is biologically based and has been reported to be independent of a patient’s demographics and smoking history [16]. Its high specificity and PPV make it a potentially complementary tool for use in conjunction with CT to evaluate a patient at high risk for lung cancer. For example, if a pulmonary nodule is identified on a CT scan and the EarlyCDT-Lung test is positive, the probability of malignancy is significantly increased (manuscript in preparation). In addition, if a patient who falls just outside the NLST criteria for CT screening tests positive by EarlyCDT-Lung, then their risk of lung cancer would be increased to a level that would now make them appropriate for CT screening. However, it is important to note that due to the relatively low sensitivity (∼41%) of EarlyCDT-Lung, a negative test result does not rule out lung cancer in either scenario; in the case of the pulmonary nodule and a negative EarlyCDT-Lung result, the physician would continue to follow the current recommendations for follow-up CT scanning per the Fleischner Guidelines [17], and in the second scenario with a negative EarlyCDT-Lung result, the physician would continue monitoring the patient’s health according to standard procedures, as they would have done in the absence of the EarlyCDT-Lung test.

Two prospective clinical trials are currently on-going – one in the US (assessing the value of the test in conjunction with CT) and a second in the UK (assessing the value of the test as a pre-CT screening tool).

5. Summary

This is the first biologically based blood test for lung cancer detection that has been extensively tested and validated in case–control settings and has now been shown to perform as predicted in clinical practice. The population on whom the test was used was high risk with 4% diagnosed with lung cancer within 6 months following EarlyCDT-Lung. A positive result on the current 7AAB EarlyCDT-Lung test was associated with a 5.4-fold increase in incidence of lung cancer compared to a negative test.

Conflict of interest statement

J.R. Jett has a research grant from Oncimmune. L.J. Peek is an employee of Oncimmune USA LLC. L. Fredericks, W. Jewell and W.W. Pingleton are consultants to Oncimmune USA LLC. J.F.R.
Robertson is Chief Scientific Officer and a shareholder of Oncimmune Ltd, a University of Nottingham spinout company.

Acknowledgements

The authors wish to acknowledge and thank the physicians and office staff who were an integral part of this project.

References


EarlyCDT®-Lung test: improved clinical utility through additional autoantibody assays

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Received: 9 February 2012 / Accepted: 12 March 2012 © The Author(s) 2012. This article is published with open access at Springerlink.com

Abstract Tumor-associated autoantibodies (AAbs) have been described in patients with lung cancer, and the EarlyCDT®-Lung test that measures such AAbs is available as an aid for the early detection of lung cancer in high-risk populations. Improvements in specificity would improve its cost-effectiveness, as well as reduce anxiety associated with false positive tests. Samples from 235 patients with newly diagnosed lung cancer and matched controls were measured for the presence of AAbs to a panel of six (p53, NY-ESO-1, CAGE, GBU4-5, Annexin I, and SOX2) or seven (p53, NY-ESO-1, CAGE, GBU4-5, SOX2, HuD, and MAGE A4) antigens. Data were assessed in relation to cancer type and stage. The sensitivity and specificity of these two panels were also compared in two prospective consecutive series of 776 and 836 individuals at an increased risk of developing lung cancer. The six-AAB panel gave a sensitivity of 39% with a specificity of 89%, while the seven-AAB panel gave a sensitivity of 41% with a specificity of 91% which, once adjusted for occult cancers in the population, resulted in a specificity of 93%. Analysis of these AAB assays in the at-risk population confirmed that the seven-AAB panel resulted in a significant increase in the specificity of the test from 82 to 90%, with no significant change in sensitivity. The change from a six- to a seven-AAB assay can improve the specificity of the test and would result in a PPV of 1 in 8 and an overall accuracy of 92%.

Keywords Autoantibodies · Lung cancer · Lung cancer diagnosis

Introduction

Patients with lung cancer, both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), can mount a humoral immune response to their cancer [1–5]. Autoantibodies (AAbs) have been described not only at the time of initial diagnosis of lung cancer [1, 2], but also, in some cases, up to 5 years before the cancer is diagnosed [6–8]. There is now level 1 evidence from the US National Lung Screening Trial that earlier diagnosis saves lives; this randomized control trial reported a 20% reduction in lung cancer mortality, following CT screening of high-risk individuals [9].
An AAb assay for lung cancer (EarlyCDT®-Lung), which was technically and clinically validated using three separate case–control study populations, has recently been reported [1, 10]. In these publications, AAbs to six tumor-associated antigens (p53, NY-ESO-1, Annexin I, CAGE, GBU4-5, and SOX2) were measured and identified up to 40% of all lung cancers in the disease groups, with a specificity of 90% (non-cancer controls individually matched to lung cancer sera by age, gender, and smoking history) [1, 10]. Further confirmation of the sensitivity and specificity of the test for lung cancer using four new, independent sample sets (n=574 newly diagnosed lung cancers plus controls) was recently reported [11], with no significant difference in positivity for EarlyCDT-Lung among different cancer stages being seen. The performance of the test (in terms of precision and analytical linearity [10]) is such that it is now commercially available to clinicians, to assist them in the early detection of lung cancer in combination with imaging techniques.

We report here the analysis of two additional and well-described cancer-associated antigens, MAGE A4 and HuD (n-ELAV), which are known to have particular associations with lung cancer. The MAGE gene family belongs to the chromosome X-clustered cancer/testis antigens, and the members of the MAGE A family encode proteins with 50 to 80% sequence identity to each other. The overexpression of these MAGE antigens has been described in a number of cancers including lung cancer [12, 13], and MAGE A4 has been proposed as a potential therapeutic target for immunotherapy [14]. The diagnostic potential of MAGE A4 AAb measurement has not been reported previously. HuD is a member of a family of onconeuronal RNA-binding proteins known for stabilizing RNA. It is normally expressed only on terminally differentiated neurons where it is involved in the development and maintenance of the nervous system [15–17]. Anti-Hu antibodies are often found associated with paraneoplastic encephalomyelitis or sensory neuropathy, and these antibodies have been described in neuroendocrine tumors of the lung, in particular SCLC [18–20]. In fact, 17% of patients with SCLC have been described as having elevated levels of AAbs to HuD when compared to matched controls [20].

This manuscript reports an improved EarlyCDT-Lung panel with the addition of these two new AAb assays (i.e., MAGE A4 and HuD), and the removal of Annexin I, first in an optimization set comprised of patients with newly diagnosed lung cancer (before any treatment) and matched controls, and secondly in a prospective sample set confirming the additive value this modification brings to the original EarlyCDT-Lung panel in the clinical setting.

Methods

Blood samples and patient details

Optimization set

Serum samples from 235 patients with lung cancer (from the UK, USA, Ukraine, and Russia), obtained at or just after histopathological confirmation of the tumor, were assayed. These 235 samples represented 87% of the lung cancers in a previously published dataset (group 3, n=269) [1], which were chosen on the basis of sufficient residual sample volume being available for analysis. The lung cancers consisted of 178 NSCLCs (75.7%), 53 SCLCs (22.6%), and 4 others (1 sarcoma, 2× bronchogenic carcinomas, and 1 undefined lung cancer). The controls were also part of the previously published sample set and consisted of 266 healthy volunteers, 235 of which were matched to the lung cancer patients for age, gender, and smoking status. This group of controls had no evidence of any current or prior cancer including non-melanoma skin cancer. All serum samples were collected following informed consent and stored at −20 or −70°C prior to analysis. This dataset was used to re-optimize the panel performance in terms of specificity following the addition of the new antigens and the removal of Annexin I.

Clinical population set

The performance of the AAb test in an independent, clinically relevant sample set is reported here using the clinical samples sent for commercial EarlyCDT-Lung measurement [1, 10]. These consisted of 776 serum samples assessed by the original six-antigen AAb assay panel (May 2009–November 2010) and a further separate but consecutive 836 serum samples assessed by the updated seven-antigen AAb assay panel (November 2010–August 2011). All samples were from individuals in North America deemed by their clinician to be at an increased risk of developing lung cancer due to age and smoking history or other factors. All sera were taken under informed consent, and all patients had signed a HIPAA release, authorizing access to their medical records.

Antigen production

Recombinant proteins were cloned into pET expression vectors (Invitrogen) and transformed into Escherichia coli BL21 (DE3) bacteria. The proteins p53, NY-ESO-1, CAGE, Annexin I, MAGE A4, HuD, SOX2-B, and GBU4-5 were cloned into pET21b and produced with a His tag and BirA tag [1, 10], whereas SOX2-N was cloned into pET44b and produced with a His tag and NusA tag. Negative control proteins were also produced (BirA and NusA tags alone).
The recombinant proteins were expressed in BL21 (DE3) bacteria (Novagen) and grown in terrific broth (TB), auto-induction TB media (Novagen), ECPM media, or Power Broth (Molecular Dimensions). Recombinant proteins were purified by metal chelate affinity chromatography and refolded by dialysis [10, 21]. All recombinant proteins were produced by external suppliers. Quality control tests for acceptance of protein included SDS–PAGE, Western blotting with appropriate antibodies, and analytical size exclusion chromatography.

Autoantibody detection

AAbs to the tumor-associated antigens were measured using EarlyCDT-Lung (Oncimmune USA LLC, De Soto, KS), a commercially available blood test based on ELISA principles that uses microtiter plates coated with a set of serial dilutions of recombinant antigens as previously described [10]. All assays were run blinded to the demographic data. AAbs were measured as optical density units and then expressed in calibrated reference units (RU). Positive seroreactivity for the assay was defined as (a) having evidence of a dose response to the antigen titration series and (b) an assay result above a cutoff level (described below).

Statistics

Assay data handling (calibration of OD signal to RU) was performed by the Oncimmune LLC LIMS system. Clinical performance was expressed in terms of sensitivity (the percentage of true positives) and specificity (the percentage of true negatives). Concordance (the percentage of samples with the same test outcome in two assays being compared), accuracy (the percentage of samples correctly diagnosed), and positive predictive value (PPV; the probability of cancer given a positive test result) were also calculated. This analysis was performed using Microsoft Excel. For comparison of sensitivity and specificity values, chi-squared tests were used. Forest plots of the sensitivity at fixed specificity for subgroups were prepared using 95% binomial confidence intervals. Similarly, for individual antigens, 95% binomial confidence intervals were calculated for percentage positivity (sensitivity). This analysis was performed using SPSS®.

Assessment of lung cancer risk

Underlying risk was calculated from the Spitz et al. [22] individual lung cancer risk assessment model, which captures some of the complex interactions between exposures and host susceptibility factors. The model was adapted to predict 5-year absolute risk of lung cancer, based on gender, age, and smoking history. An in-house program was used for the calculations [23].

Optimization of assay cutoffs

A fixed target specificity of 90% was selected for the panel of six AAb assays, and the cutoffs were obtained by optimizing sensitivity using a Monte Carlo direct search method [24] and validated as previously described [1, 10]. The method searches a random selection (n=10,000) of the possible sets of cutoffs and chooses the set with the highest sensitivity for the fixed specificity. For the new panel of seven assays (including the two new antigens and SOX2-B and the removal of Annexin I), a similar Monte Carlo approach was used but this time optimizing specificity for a fixed sensitivity of approximately 40%. The optimization was performed using R software.

Adjustment for lung cancers in the control populations

In order to set accurate and meaningful cutoffs for lung cancer detection tests, the results obtained from a group of individuals known to have the disease must be compared with those obtained from a group of individuals with demographic and risk factors matched to the cancer group and known to be disease free. However, obtaining a truly disease-free control group is extremely problematic since CT screening studies have shown that in any high-risk group there is a small proportion of individuals harboring undiagnosed asymptomatic lung cancer [9]. The proportion of such individuals may be as high as 2.7% in a prevalence round and 2.3% in an incidence round (referenced in [1]). For this reason, a modified lung cancer prediction model [22] was employed that allowed for the presence of occult cancers in the control population by taking into account the most important predictors for disease such as smoking status and history as well as age. The adjustment was carried out in the Monte Carlo optimization routine as described previously [1, 24] to provide accurate sensitivity and specificity values for the EarlyCDT-Lung test.

Results

Optimization set

The sensitivity and specificity of the AAb assays for 235 lung cancers are shown in Table 1 where the data are also characterized by tumor type (i.e., NSCLC and SCLC), and a summary of the demographics of the population is shown in Table 2.

Elevated AAb levels to at least one of the original six antigens in the EarlyCDT-Lung test (p53, CAGE, NY-ESO-1, GBU4-5, Annexin I, and SOX2-N), using the original published cutoffs, gave an overall sensitivity for lung cancer detection of 39% with an unadjusted specificity of 89%, while...
Specificity for lung cancer detection in the normal population is also shown. Specificity is unadjusted for the presence of cancers in the control population.

Data are shown as percentage positivity following the application of the adjusted cutoffs. Numbers in parentheses are the 95% confidence interval.

| Specificity | 266 | 99 | 97 | 99 | 98 | 98 | 99 | 99 | 99 | 91 |

MAGE A4, SOX2-B, and HuD

Normals 266 0 (0–266) 0

NSCLC 53 0 (0–21) 0

SCLC 53 0 (0–21) 0

The detection of AAbs to some antigens was, however, more specific for the detection of certain cancer subtypes; for example, MAGE A4 predicted the presence of NSCLC more often than SCLC, while the reverse was true for HuD and SOX2 (Table 1).

Allowing for the presence of potentially undiagnosed cancers in the high-risk control population (as described above), the seven-AAb test demonstrated an adjusted specificity and sensitivity of 93 and 41%, respectively. This would mean that in a high-risk population (e.g., lung cancer prevalence of 2.4% [25]), such a change in the panel would result in an improvement in the PPV of the test from 9 (1 in 11) to 13% (1 in 8) and therefore an increase in the accuracy of the test from 89 to 92%. For comparison, if a lower prevalence of lung cancer is assumed (e.g., 1.3% [26]), the PPV of the new EarlyCDT-Lung (seven-assay test) test would be 7% (1 in 14) with an accuracy of 92%.

Clinical population set

The performance of the assay was evaluated in a prospective series of individuals at increased risk of developing lung cancer, by auditing the clinical follow-up data alongside the EarlyCDT-Lung results for 1,612 clinical samples, run sequentially either on the original panel of six-AAb assays (776 samples) or the new panel of seven-AAb assays (836 samples). The two sets of commercial samples could not be analyzed by both the original and new panels so direct comparison of sensitivity and specificity could not be performed.

The demographics of the two groups were similar in terms of mean age and range; however, the proportion of men was higher in the six-AAb assay group, as was the average risk for development of a lung cancer (Table 3).

Overall, 2.7% of these individuals (44/1,612) were diagnosed with lung cancer after having the EarlyCDT-Lung test. When the lung cancer diagnosis was analyzed according to whether the individuals were tested using the six- or seven-AAb test, 3.2% of those who were tested using the six-AAb test and 2.3% of those who were tested using the seven-AAb test showed AAb positivity to any one of the antigens in the new seven-AAb EarlyCDT-Lung panel: p53, CAGE, NY-ESO-1, GBU4-5, MAGE A4, SOX2-B, and HuD.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sensitivity and specificity of AAb assays for the optimization set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>All LCa</td>
<td>235</td>
</tr>
<tr>
<td>NSCLC</td>
<td>178</td>
</tr>
<tr>
<td>SCLC</td>
<td>53</td>
</tr>
<tr>
<td>Normals</td>
<td>266</td>
</tr>
</tbody>
</table>

Data are shown as percentage positivity following the application of the adjusted cutoffs. Numbers in parentheses are the 95% confidence interval. Specificity for lung cancer detection in the normal population is also shown. Specificity is unadjusted for the presence of cancers in the control population. Panel of 7 represents AAb positivity to any one of the antigens in the new seven-AAb EarlyCDT-Lung panel: p53, CAGE, NY-ESO-1, GBU4-5, MAGE A4, SOX2-B, and HuD.

Table 2 | Demographics of the optimization data set |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic data</td>
<td>Cancer sera</td>
</tr>
<tr>
<td>Number</td>
<td>235</td>
</tr>
<tr>
<td>Male/female</td>
<td>73%/27%</td>
</tr>
<tr>
<td>Age mean (median)</td>
<td>64.8 (65)</td>
</tr>
<tr>
<td>Current smoker/ex smoker</td>
<td>46%/29%</td>
</tr>
<tr>
<td>Nonsmoker/unknown</td>
<td>10%/15%</td>
</tr>
</tbody>
</table>

Both cancer and normal sera were analyzed using both the six- and seven-AAb panel of assays. Normal sera were matched as closely as possible from the available samples to the cancer sera for age, gender, and smoking history.
AAb test had developed lung cancer, reflecting the increased risk calculated for the earlier group (Tables 3).

Of the 44 individuals diagnosed with lung cancer, 19 had elevated levels of AAbs, and the panel identified SCLC

**Fig. 1** Forest plot showing the sensitivity of the EarlyCDT-Lung assay at a fixed specificity of 93% (with confidence intervals) by tumor characteristics and lung cancer stage. Positivity is defined as having an elevated AAb assay signal to any one of the antigens in the new seven-AAb EarlyCDT-Lung panel: p53, CAGE, NY-ESO-1, GBU4-5, MAGE A4, SOX2-B, and HuD. Vertical dashed line represents sensitivity at 40% (all stages of lung cancer). NSCLC non-small cell lung cancer, SCLC small cell lung cancer, LD limited disease, ED extensive disease, early stage stage I and II NSCLCs and LD SCLCs, late stage stage III and IV NSCLCs and ED SCLCs. The number of samples in each group is represented in parentheses.

### Table 3 Demographics of the population data sets

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Number</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 AAb</td>
<td>776</td>
<td>Male 48%, female 52%</td>
</tr>
<tr>
<td>7 AAb</td>
<td>836</td>
<td>Male 36%, female 64%</td>
</tr>
<tr>
<td>Total</td>
<td>1,612</td>
<td>Male 42%, female 58%</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>Mean ([5%ile]–median–(95%ile])</td>
</tr>
<tr>
<td>6 AAb</td>
<td>776</td>
<td>61 [(45)–62–(77)]</td>
</tr>
<tr>
<td>7 AAb</td>
<td>836</td>
<td>60 [(43)–59–(79)]</td>
</tr>
<tr>
<td>Total</td>
<td>1,612</td>
<td>61 [(44)–61–(78)]</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td>Percentage</td>
</tr>
<tr>
<td>6 AAb</td>
<td>721</td>
<td>Caucasian 92.0%, Afr-Amer 5.7%, Hispanic 1.7%, Others 0.6%</td>
</tr>
<tr>
<td>7 AAb</td>
<td>811</td>
<td>Caucasian 90.6%, Afr-Amer 5.2%, Hispanic 2.6%, Others 1.6%</td>
</tr>
<tr>
<td>Total</td>
<td>1,532</td>
<td>Caucasian 91.3%, Afr-Amer 5.4%, Hispanic 2.2%, Others 1.1%</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td>Percentage</td>
</tr>
<tr>
<td>6 AAb</td>
<td>770</td>
<td>Current 47.0%, ex smoker 48.3%, nonsmoker 4.7%</td>
</tr>
<tr>
<td>7 AAb</td>
<td>836</td>
<td>Current 43.4%, ex smoker 44.3%, nonsmoker 12.3%</td>
</tr>
<tr>
<td>Total</td>
<td>1,606</td>
<td>Current 45.1%, ex smoker 46.2%, nonsmoker 8.7%</td>
</tr>
<tr>
<td>Lung cancer risk&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>Mean [min–(5%ile)–median–(95%ile)–max]</td>
</tr>
<tr>
<td>6 AAb</td>
<td>770</td>
<td>3.1 [0.0–(0.0)–2.7–(8.3)–11.9]</td>
</tr>
<tr>
<td>7 AAb</td>
<td>836</td>
<td>2.4 [0.0–(0.0)–1.6–(7.3)–11.9]</td>
</tr>
<tr>
<td>Total</td>
<td>1,606</td>
<td>2.7 [0.0–(0.0)–2.1–(8.0)–11.9]</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number denotes numbers for which data were available

<sup>b</sup>Lung cancer risk was calculated according to a modified Spitz et al. lung cancer prediction model [22] based on gender, age, and smoking history
Table 4: Audit of EarlyCDT-Lung test

<table>
<thead>
<tr>
<th>Panel of 6-AAb assays</th>
<th>Number of participants</th>
<th>Confirmed lung cancersa, N (%)</th>
<th>No lung cancer diagnosisb, N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>776</td>
<td>25 (3.2)</td>
<td>751 (96.8)</td>
</tr>
<tr>
<td>Positive AAb assay result</td>
<td>145</td>
<td>10 (6.9)</td>
<td>135 (93.1)</td>
</tr>
<tr>
<td>Negative AAb assay result</td>
<td>631</td>
<td>15 (2.4)</td>
<td>616 (97.6)</td>
</tr>
<tr>
<td>Overall panel sensitivity or specificity</td>
<td></td>
<td>Sensitivity 40%</td>
<td>Specificity 82%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel of 7-AAb assays</th>
<th>Number of participants</th>
<th>Confirmed lung cancersa, N (%)</th>
<th>No lung cancer diagnosisb, N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>836</td>
<td>19 (2.3)</td>
<td>817 (97.7)</td>
</tr>
<tr>
<td>Positive AAb assay result</td>
<td>87</td>
<td>9 (10.3)</td>
<td>78 (89.7)</td>
</tr>
<tr>
<td>Negative AAb assay result</td>
<td>749</td>
<td>10 (1.3)</td>
<td>739 (98.7)</td>
</tr>
<tr>
<td>Overall panel sensitivity or specificity</td>
<td></td>
<td>Sensitivity 47%</td>
<td>Specificity 90%</td>
</tr>
</tbody>
</table>

*Original six-AAb assay panel (performed on 776 samples) and new seven-AAb assay panel (performed on 836 samples) showing the number of samples that were identified as being positive or negative in the EarlyCDT-Lung test and the number of confirmed cases of lung cancer. Panel of 6 represents AAb positivity to any one of the original six-AAb EarlyCDT-Lung panel: p53, CAGE, NY-ESO-1, GBU4-5, Annexin I, and SOX2-N. Panel of 7 represents AAb positivity to any one of the new seven-AAb EarlyCDT-Lung panel: p53, CAGE, NY-ESO-1, GBU4-5, MAGE A4, SOX2-B, and HuD.

a Number of lung cancers detected—correct as of August 2011 following CT and biopsy.
b Number of individuals assessed as being free from lung cancer, as of August 2011.

(1/2) and NSCLC (18/42), as well as both asymptomatic early-stage (stage IA and IB) and later-stage disease. Using the original panel of six-AAb assays and original cutoffs generated in our previous publication [10], the specificity/sensitivity in the first set of 776 samples was 82%/40%. Using the new panel of seven-AAb assays and the cutoffs established in the optimization set, the specificity/sensitivity in the second set of 836 samples was 90%/47% (Table 4). This change from the original six-AAb to the new seven-AAb panel represented a significant improvement in the specificity of the test for cancer detection ($p<0.0001$) with no significant difference between the sensitivity of the two panels ($p=0.63$; probably due to small numbers). Assuming the calculated risk of developing lung cancer for each group was 3.1 and 2.4%, respectively (Table 3), this would confer an increase in the PPV of the test from 1 in 15 to 1 in 10.

Discussion

Previous publications using validated, calibrated assays have confirmed the utility of measuring AAbs to tumor-associated antigens as an aid for the identification of early-stage lung cancers [1, 11]. The data presented in this manuscript reveal that improvements of such a test can be achieved by adding two new antigens and dropping one (now redundant) antigen from the EarlyCDT-Lung panel, and re-optimizing the cutoffs. This change essentially maintained the previously reported 40% sensitivity of the test for lung cancer [1] even for early-stage more treatable disease. Importantly however, it improved the specificity of the test (once adjusted for occult cancers in the population) from 90% as previously reported [1] to 93% in the same retrospective case–control (optimization) set. In a clinical setting, such an improvement would result in an increase in the PPV of the test and a 30% reduction in “false” positive tests, important benefits to both patients and clinicians.

Since the two additional antigens were added to ultimately increase the specificity of EarlyCDT-Lung test, it was deemed appropriate to report the performance of the test in a clinical setting, where individuals at an increased risk of developing lung cancer were tested. Data from an audit of the first 1,612 samples run on the EarlyCDT-Lung test revealed that the performance of the test was as expected in a clinically relevant group of individuals at an increased risk of developing lung cancer, and the clinical results mirrored differences in the actual (as of August 2011) and calculated (Spitz model [22]) risk between the two groups. A difference in the gender proportion between the two clinical groups was noted; however, there are no reports of differences in autoantibody levels in individuals with lung cancer between genders [1]. Furthermore, a recent study of the demographics of normal individuals also showed no difference in autoantibody levels due to gender or ethnicity in a normal group [27].

Analysis of the performance of the EarlyCDT-Lung test in the clinical population dataset showed that the sensitivity of the test for lung cancer, reported in the optimization set, was maintained in the clinical setting, where at least 40% of the lung cancers had a positive test. Although the number of lung cancers in the audit was relatively small, both panels were successful in detecting early-stage disease.
The greatest impact seen with the new seven-AAb panel was the highly significant improvement in the specificity of the test in the clinical setting. While in the retrospective case–control set the improvement in the assay specificity resulted in a 30% reduction in false positives, in the prospective clinical audit data, the change to the seven-AAb panel resulted in a 44% reduction in the number of “false positive” tests. This is because in the clinical population, the specificity of the six-AAb panel was lower than expected at 82%, while the seven-AAb panel revealed a specificity of 90% (unadjusted for occult cancers), a level similar to that predicted from the optimization dataset. An individual predicted to be at an increased risk of lung cancer due to demographic risk factors including smoking history, gender, and age, and who then had a positive EarlyCDT-Lung test, would be at a higher risk for harboring lung cancer than predicted; with the introduction of the seven-AAb version of the test, this increase in risk is even greater.

The seven-AAb test with a specificity/sensitivity of 93%/41% in a high-risk population (e.g., prevalence of 2.4% [25]) has an overall accuracy of 92% compared to approximately 50% for CT [28]. The authors, however, view AAb technology and CT imaging as being complementary rather than competitive and that the presence of AAbs may provide an aid to early detection of lung cancer, particularly in early-stage disease which is potentially curable. This improved test may therefore prove useful in the management of high-risk individuals.

Acknowledgments This work was supported by funding from Oncimmune Ltd and the University of Nottingham.

Conflicts of interest GFH, AM, JA, CBPK, and IKM are employed by Oncimmune Ltd and LJF is employed by Oncimmune LLC. JFRR consults for and is a shareholder in Oncimmune Ltd. GHF is a shareholder and chairman of Oncimmune Ltd. CJC, WJ, and CR consult for Oncimmune Ltd.

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References


Clinical validation of an autoantibody test for lung cancer

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Received 1 April 2010; revised 28 May 2010; accepted 31 May 2010

Background: Autoantibodies may be present in a variety of underlying cancers several years before tumours can be detected and testing for their presence may allow earlier diagnosis. We report the clinical validation of an autoantibody panel in newly diagnosed patients with lung cancer (LC).

Patients and methods: Three cohorts of patients with newly diagnosed LC were identified: group 1 (n = 145), group 2 (n = 241) and group 3 (n = 269). Patients were individually matched by gender, age and smoking history to a control individual with no history of malignant disease. Serum samples were obtained after diagnosis but before any anticancer treatment. Autoantibody levels were measured against a panel of six tumour-related antigens (p53, NY-ESO-1, CAGE, GBU4-5, Annexin 1 and SOX2). Assay sensitivity was tested in relation to demographic variables and cancer type/stage.

Results: The autoantibody panel demonstrated a sensitivity/specificity of 36%/91%, 39%/89% and 37%/90% in groups 1, 2 and 3, respectively, with good reproducibility. There was no significant difference between different LC stages, indicating that the antigens included covered the different types of LC well.

Conclusion: This assay confirms the value of an autoantibody panel as a diagnostic tool and offers a potential system for monitoring patients at high risk of LC.

Key words: autoantibodies, clinical validation, lung cancer, newly diagnosed patients

introduction

Lung cancer (LC) is the worldwide leading cause of cancer-related mortality [1]. Tobacco smoking is estimated to cause upwards of 90% of cases, and other recognised risk factors include passive smoking, occupational exposure, especially to asbestos and radon exposure [1]. Outcomes are substantially better with early localised disease compared with locally advanced and metastatic disease, with 5-year survival rates of 53%, 23.7% and 3.5%, respectively [2].

Although the latent period of LC in smokers is reported to be at least 20 years [1], ~85% of patients with LC remain undiagnosed until the disease is symptomatic and has reached an advanced stage [2]. At present, there is nothing to offer for early diagnosis, although ongoing clinical trials are investigating the use of spiral computed tomography (CT) in ‘at-risk’ individuals [3–12]. However, the radiation dose delivered and the substantial costs limit its widespread application as a screening procedure [13]. Furthermore, the high rate of false positives (as high as 50% in a prevalence round) [5] dictates that many individuals require follow-up examinations and a substantial proportion of individuals undergo unnecessary thoracotomy [14]. Application of a filter such as a blood-based marker to identify smokers at the highest risk for LC may improve the positive predictive value (PPV) of these screening tools [11, 15].

There is a considerable body of evidence documenting the presence of circulating antibodies to autologous cellular antigens [referred to as tumour-associated antigens (TAA)] in serum samples from patients with a variety of cancers, including LC [16–24]. Monitoring persons at increased risk of cancer for the presence of serum autoantibodies may allow earlier detection of the disease.

The panel of proteins selected for investigation comprised a number of well-recognised TAA, four of which (p53, NY-ESO-1, CAGE and GBU4-5) have been described by ourselves in a previous publication to induce the production of autoantibodies or immune biomarkers in LC [24]. In brief, p53 is a tumour suppressor gene, which is often mutated in cancer and to which autoantibodies were first described [25],

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autoantibodies to this protein have also been detected in some cases, even before the cancer diagnosis [26, 27]. NY-ESO-1 and CAGE are both cancer testis antigens whose expression has been described in a number of solid tumours [28, 29] and with GBU4-5, a protein of unknown function that encodes a DEAD box domain, have also been described as inducing autoantibodies in LC [24, 30, 31].

The remaining antigens SOX2, a member of the SOX family of proteins that is described as inducing an autoantibody response in small-cell lung cancer (SCLC) [32, 33], and Annexin 1, a phospholipid-binding protein to which autoantibodies, have also been described [18].

The selection of these antigens was confirmed following screening of a panel of >20 potential antigens as being of greatest diagnostic utility for the diagnosis of all non-small-cell lung cancer (NSCLC) and SCLC cancer (C. Chapman, unpublished observations).

This manuscript reports the clinical validation set for these autoantibodies in the serum of patients with newly diagnosed LC (before any treatment) and matched controls.

patients and methods

patients

Findings from three separate groups of patients with newly diagnosed LC are reported. The third group is the final validation set where the data were run in a blinded manner. All patients with LC were as far as possible individually matched by gender, age and smoking history to a control individual with no previous history of malignant disease. In patients with LC, blood samples were obtained after diagnosis but before receiving any anticancer treatment. Demographic characteristics of the control versus the study population are given in the Appendix 1.

Group 1 comprised 145 patients with stage I/II LC (including NSCLC and SCLC) and 146 controls treated in centres in the United States and Russia. All subjects in this group were smokers; baseline patient characteristics are shown in Table 1. Group 2 comprised 241 patients with LC treated at a single centre in Germany as part of a collaborative study (Table 2). Tumour pathological information was available for the patients with LC, including tumour, node, metastasis staging and NSCLC histology (Table 2). In group 2, an additional 88 sera from unmatched individuals (25 normal and 63 with benign lung conditions) supplied by the single centre were analysed (Appendix 1).

Group 3 comprised 269 patients with LC treated at centres in the United States, UK and Ukraine (Table 1). This group was assembled to validate the calibration and control scheme for the autoantibody assay. Tumour pathological information was available for the patients with LC (Table 2). The timeline for collection of samples from patients is shown in supplemental Table S1 (available at Annals of Oncology online).

Serum samples in group 1 were evaluated for autoantibodies against p53, NY-ESO-1, CAGE and GBU4-5. Serum samples in groups 2 and 3 were evaluated for autoantibodies against the same four antigens plus Annexin 1 and SOX2. In groups 2 and 3, samples from patients with cancers, matched normals, benign lung disease and control sera for the assay were interspersed in the order samples were assayed so that any batch effects would be spread over all sample types. The laboratory staff running the assay was blinded to the disease state of individual samples. Group 2, therefore, was a validation set for the results seen in group 1 for four of the antigens (i.e. p53, NY-ESO1, CAGE and GBU4-5) with the added value of Annexin 1 and SOX2. Group 3 validated a calibrated and controlled assay on the whole panel of six antigens.

autoantibody assay

Autoantibodies were determined by a quality-controlled, semi-automated indirect enzyme-linked immunosorbent assay in which samples were allowed to react with a titration series of antigen concentrations. All liquid handling steps were carried out using an automated liquid handling system. Briefly, purified recombinant candidates were diluted to provide a semi-log titration series for each antigen from 160 to 1.6 nM [34]. Control antigens consisting of the purified BirA or NusA tags were also included to allow subtraction of the signal due to nonspecific binding to bacterial contaminants. Antigen dilutions were adsorbed to the surface of microtitre plate wells in phosphate buffer at room temperature. After washing in phosphate-buffered saline containing 0.1% Tween 20 (pH 7.6), microtitre plates were blocked with a gelatine-based blocking buffer. Serum samples (diluted 1 in 110 in a blocking buffer) were then added to the plates and allowed to incubate at room temperature with shaking for 90 min. Following incubation, plates were washed and horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark) was added. After a 60-min incubation, the plates were washed and 3,3′,5,5′-tetramethylbenzidine was added. Colour formation was allowed to proceed for 15 min before the optical density (OD) of each well was determined spectrophotometrically at 650 nm [35].

Calibration standards of known potency are not available for assays to measure autoantibodies against TAAs. Therefore, a calibration system was devised which utilised fluids drained from pleural or ascitic cavities of patients with LC [36]. The calibration system was only evaluated for group 3 samples. A reportable dilution range for each antigen, giving acceptable calibration precision, was determined at 7.5%–92.5% of the upper asymptote of the average calibration curve, equivalent to ~5.0 natural log reference units (RU). These data were used to construct a calibration curve of OD versus log dilution to which a four-parameter model plot was fitted [37]. The background-corrected OD value for each unknown sample was then converted to a calibrated log RU.

Samples were judged to be positive if they fulfilled two criteria—i.e. they showed a dose response to the antigen titration series and the measured autoantibody signal to one or more of the antigens was above the accepted cut-off set for that antigen assay. The autoantibody signal for a sample was defined as above the cut-off when the result was greater than the calculated cut-off for the control population at either of the two highest points on the titration curve. All assays were carried out as two replicates and the mean value taken as the overall assay measurement.

optimisation of assay cut-offs

A specificity of 90% was selected in order to produce a test which could be used for early detection in a high-risk population and which would be health economically viable. For all groups, cut-offs based on mean + 3

Table 1. Lung cancer patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n = 145)</th>
<th>Group 2 (n = 241)</th>
<th>Group 3 (n = 269)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td>66 (41–87)</td>
<td>63 (28–87)</td>
<td>65 (58–87)</td>
</tr>
<tr>
<td>Patients &gt;60 years, n (%)</td>
<td>96 (66.2)</td>
<td>140 (58.1)</td>
<td>171 (63.6)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>81 (55.9)</td>
<td>172 (71.4)</td>
<td>199 (74.0)</td>
</tr>
<tr>
<td>Female</td>
<td>64 (44.1)</td>
<td>69 (28.6)</td>
<td>70 (26.0)</td>
</tr>
<tr>
<td>Smoking history, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>145</td>
<td>0</td>
<td>132 (49.1)</td>
</tr>
<tr>
<td>Previous</td>
<td>0</td>
<td>0</td>
<td>76 (28.3)</td>
</tr>
<tr>
<td>Never</td>
<td>0</td>
<td>0</td>
<td>24 (8.9)</td>
</tr>
<tr>
<td>Not determined</td>
<td>0 (0.0)</td>
<td>241 (100.0)</td>
<td>37 (13.8)</td>
</tr>
</tbody>
</table>
standard deviations (SDs) of the normal population were used. In addition, for groups 2 and 3, the cut-offs were optimised using a Monte Carlo direct search method [38] to find a set of antigen-specific cut-offs yielding the maximum sensitivity for the fixed specificity of 90%.

For a set of possible cut-offs for the six panel antigens chosen by Monte Carlo sampling over the feasible range, the specification/sensitivity was first estimated from the data. This was carried out 100 000 times. All combinations with a specificity of ~90% were then extracted and the combination yielding the maximum sensitivity used. This is a process dependent on assay conditions and when new batches of proteins, or new types of protein, are introduced to the panel, new cut-offs will have to be calculated.

To support the quoted specification/sensitivity panel results, the area under the curve (AUC) and standard error (SE) for the respective receiver operating characteristic (ROC) curve was calculated for each group. The ROC curve was constructed by calculating the specification and sensitivity of the test for a succession of deviations from the original cut-offs, with the same deviation for each antigen in the panel.

### adjustment for LCs in the control populations

The cut-offs are best set by comparing the results in a group of patients with known LC and a group of high-risk individuals (e.g. smokers and ex-smokers) who are known to not have the disease. However, the latter population is difficult to identify since the CT screening studies have clearly shown there are a percentage of smokers/ex-smokers who at any one time are ‘harbouring’ an asymptomatic LC. In the prevalence round, the percentage of undiagnosed occult cancers has been reported to be between 0.5% and 2.7% in heavy smokers, while in incidence rounds, it has been reported to be up to 2.3% [3–12]. For this reason, adjusted specificity and sensitivity values assuming some degree of occult LCs in the control populations were also calculated.

### results

#### autoantibody expression

In group 1, autoantibodies to four antigens (p53, NY-ESO-1, CAGE, and GBU4-5) were measured as raw OD values. Using cut-offs based on mean + 3 SDs gave a sensitivity of 36% with a specificity of 91% (50 of the 137, 8 unassessable). The sensitivities and specificities for each of these four antigens and the reproducibility of these assays have been reported elsewhere [35]. The sensitivity and specificity of the panel was similar for males and females. The ROC curve AUC was 0.71 (SE = 0.03).

In group 2, autoantibodies to six antigens (p53, NY-ESO-1, CAGE, GBU4-5 plus Annexin 1 and SOX2) were measured as raw OD values, with cut-offs based on mean + 3 SDs producing sensitivities and specificities for these six antigens have been reported elsewhere [35]. Using individually optimised cut-offs for each antigen, the overall sensitivity was 39% (33%–45%) (91 of the 234), with a specificity of 89%. In an at-risk population of 20 LCs per 1000 population, this would result in a PPV of 7.2% (i.e. 1 in 13.9 persons with a positive test would have a LC) and a negative predictive value (NPV) of 98.6%. The ROC curve AUC was 0.63 (SE = 0.03).
Of the 88 unmatched sera received from the group 2 centre, 8 of the 88 (9%) were positive, of which none of the 25 (0%) normal sera had raised autoantibodies, while 8 of the 63 (13%) of individuals with benign lung disease had raised autoantibodies. Follow-up data could only be obtained for one of these eight individuals who was found to have developed a gastric cancer, giving a specificity of at least 89% (35 of the 62).

In group 3, autoantibodies to the same six antigens as group 2 were measured as raw OD values and converted into calibrated RU. Using the ODs and applying cut-offs based on mean + 3 SDs gave a sensitivity of 32% (85 of the 269) with a specificity of 91%. Using RU values with individually optimised cut-offs for each antigen, the sensitivity was 37% (100 of the 269), with a specificity of 90%. In an at-risk population of 20 LCs per 1000 population, this would result in a PPV of 7.0% (i.e. 1 in 14.3) and an NPV of 98.6%.

The ROC curve AUC was 0.64 (SE = 0.02).

Individual antigen sensitivity and specificity are shown in supplemental Table S2 (available at Annals of Oncology online).

**adjustment for occult LCs within the control population**

Adjustment generated specific cut-offs for each antigen for the different methods. The sensitivities for each antigen for a fixed specificity of 90% are shown for the unadjusted and adjusted method in Table 3. The most conservative estimate for adjusted sensitivity is 40%, which in an at-risk group of 20 LCs per 1000 population would give a PPV of 7.5% (i.e. 1 in 13.3) and an NPV of 98.7%.

**effect of patient and disease characteristics on autoantibody assay sensitivity and specificity**

The calibrated group 3 dataset with an unadjusted sensitivity and specificity of 37% and 90%, respectively, was used to assess whether patient characteristics, tumour type or stage gave rise to significant variation in the specificity/sensitivity (Figure 1). Statistical comparison of subgroups with remaining controls demonstrated no significant difference in sensitivity according to patient gender, smoking status and age or tumour type or stage (P > 0.10). There was also no significant difference in sensitivity between those NSCLC tumours where the subtype was known and those where it was unknown.

**discussion**

This report confirms a validated assay for the detection of autoantibodies to selected cancer-associated antigens in the peripheral blood. The value of a test for early cancer detection is usually defined via a number of related parameters, including sensitivity, specificity, PPV and NPV. A percentage of smokers/ex-smokers are ‘harbouring’ an asymptomatic LC at any one time. Even with the most conservative estimation of occult LCs, the panel of autoantibodies can identify 40% of primary LCs, including early stage of disease, with a specificity of 90% against age-matched, gender-matched and smoking history-matched controls. The specificity was similar (at least 89%) for patients with benign disease.

**Table 3. Comparison of performance before and after adjustment for the presence of undiagnosed occult cancers in the control population**

<table>
<thead>
<tr>
<th>Adjustment method</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>Sensitivity 39% (91/234) 37% (100/269)</td>
<td>Specificity 89% (207/232) 90% (242/269)</td>
</tr>
<tr>
<td>Occult cancer rate (5%)</td>
<td>Sensitivity 42% (99/234) 40% (108/269)</td>
<td>Specificity 90% (197/220) 90% (230/255)</td>
</tr>
<tr>
<td>Occult cancer rate (11%)</td>
<td>Sensitivity 46% (108/234) 43% (115/269)</td>
<td>Specificity 89% (184/206) 90% (214/238)</td>
</tr>
</tbody>
</table>

Sensitivity for specificity of 90% ± 1%, based on optimised cut-offs for individual antigens.

Autoantibodies to p53 [26, 27, 40, 41], NY-ESO-1 [30, 31], CAGE [29, 42], GBU-4-5 [31], Annexin 1 [16, 18, 43] and SOX2 [44] have all been shown to be capable of inducing autoantibodies in patients with LC. The data in this manuscript further confirm the value of a panel of autoantibodies over a single autoantibody assay [19, 23, 24, 35]. Recent publications have reported autoantibodies to a natural form of Annexin 1 [43] and other antigens (e.g. 14-3-3 theta [43, 45] and LAMR1 [43]), which are elevated in LC and up to 1 year before clinical diagnosis. The combination of 14-3-3 theta, Annexin 1 and LAMR1 gave an AUC on a combined ROC curve of 0.73. While these results were based on a research assay, it is possible that adding 14-3-3 theta and/or LAMR1 to the current panel might increase the sensitivity.

Group 3 data confirm that there was no significant difference between different stages of LC, although due to sample size the confidence intervals were sometimes wide. Further evaluation of the data was, therefore, carried out by comparing early-stage (stage I/II NSCLC plus limited SCLC) with late-stage (stage III/IV NSCLC plus extensive SCLC) disease, which again showed no difference. The presence of such a signal in early-stage disease is precisely what would be expected of an *in vivo* amplification signal such as the humoural immune response.
This is in contrast to cancer-associated antigens, which are markers of tumour burden and not useful for the early detection or screening of breast [46, 47] or colorectal cancer [48, 49].

Previous publications [16–24, 50] have highlighted the potential value of a panel of autoantibodies for the early detection of cancer. Using a panel of antigens, autoantibodies have been reported up to 5 years before screening CT scans [22] in LC and up to 4 years before screening mammography in young women at increased risk [21, 23]. Other authors have highlighted individual autoantibodies such as p53 autoantibodies detected before diagnosis of cancer in smokers with chronic obstructive pulmonary disease [27] or in patients with asbestosis [41]. In the latter publication, the average lead time (time from first positive sample to diagnosis) was 3.5 years (range 1–12 years). There are similar publications on other single autoantibodies [45, 51, 52]. These findings all indicate the induction of autoantibodies happening relatively early in the process of carcinogenesis.

This panel assay is the first to show reproducible results with a calibration and control system and offers a potential system for monitoring a population at high risk of LC, either alone or in conjunction with imaging modalities (e.g. CT). The similar sensitivities and specificities measured for these three datasets and with different batches of proteins utilised emphasise the robustness of these autoantibody assays and also confirm the value of a panel of autoantibodies over a single autoantibody assay.

At a fixed 90% specificity, the sensitivity of 40% is a conservative estimate of the performance of the assay both in terms of estimating the level of clinically occult LCs (supplemental Table S2, available at Annals of Oncology online) and also the sensitivity reported for SCLC \( (n = 73) \) in group 3. The latter is lower than the 55% sensitivity and 90% specificity, which the authors will report in a larger consecutive series \( (n = 242) \) from a single centre (C. J. Chapman, A. J. Thorpe, A. Murray et al., unpublished data).

The sensitivity of 40% with a specificity of 90% are similar to mammography in high-risk young women [53], while the incidence of LCs in heavy smokers is at least three times the incidence of breast cancer in a typical cohort of high-risk young women [5, 7]. Therefore, in terms of absolute number of cancers, this test should detect more LCs for every 1000 high-risk persons tested than screening mammography would detect breast cancers in a high-risk group of young women, even if mammography were 100% sensitive rather than its current 40% [53]. This has to be seen in the context of a disease (i.e. LC), which has a mortality rate between 85% and 95%. By way of contrast, annual CT in the Mayo CT screening trial had a specificity of 49% (with a sensitivity of 67%) in the prevalence round. In an at-risk group of 20 of the 1000, CT gave a PPV of 2.5% (i.e. 1:40) and an NPV of 98.7%. The autoantibody test with a sensitivity of 40% and a specificity of 90% would have a PPV of 7.3% (i.e. 1 in 13.3) and an NPV of 98.7% in a similar-risk group.

While such comparisons serve to highlight the potential value of an autoantibody test for LC that has a specificity of 90%, the authors envisage the autoantibody technology and imaging as being complementary.

**funding**

Oncimmune Ltd and the University of Nottingham.

**acknowledgements**

The authors would like to thank Petra Stieber and Joachim von Pawel for their role in the collection of samples and data included in this study. They also acknowledge Céline Parsy-Kowalska’s role in protein production and Jared Allen’s contribution as the data manager. The manuscript was drafted by the authors. However, the authors acknowledge and thank Sandra Cusco, PhD, from Complete Medical Communications, who provided final editorial assistance funded by Oncimmune Ltd and the University of Nottingham.

**disclosure**

CJC and JFRR are consultants to Oncimmune Ltd, a University of Nottingham spinout company and JFRR holds stock. AM and GH are full-time employees of Oncimmune Ltd. CR holds stock option and is also a consultant to Oncimmune Ltd. WCCW is the scientific advisor for Oncimmune Ltd. GH is an employee of Oncimmune Ltd. GHF is the Chairman of Oncimmune Ltd and holds stock. ACB holds stock and options in Oncimmune Ltd and has a significant conflict.

**references**


appendix 1

demographic characteristics of the control versus the study population

A total of 655 lung cancer (LC) sera (476 were from patients with non-small-cell lung cancer, 165 with small-cell lung cancer, 1 lung sarcoma and 13 of unknown histology) were compared directly with 655 normal sera, which were analysed as controls. In addition, sera from 88 unmatched individuals (25 normal and 63 with benign lung conditions) supplied by the group 2 centre were analysed as controls to check the positivity rate in known benign lung disease. Samples were obtained, with full informed consent, at different sites. Controls for patients in group 1 were matched on the basis of gender and age (±4 years). As all subjects in this group were smokers, pack-year matching was attempted, but a tight match was prohibited by lack of information. There were 81 males and 64 females in the LC group and 83 males and 62 females in the
control group. The median age (range) of the LC patients and controls were 66 (41–87) and 66 (41–87) years, respectively. In group 2, there were 172 males and 69 females in the LC group and 171 males and 69 females in the control group. The median age (range) of the LC patients and controls were 63 (28–87) and 63 (28–87) years, respectively. Controls for group 2 were selected from a prospective collection of blood samples taken from a larger sample set of a normal population in the Midlands of England. Patients with LC were initially matched to controls on the basis of gender, age (±3 years) and smoking history. In <5% of cases, these criteria could not be met, so a choice had to be made to either extend the age-match criteria or ignore the gender-match stipulation. Since the authors have never observed a significant gender difference, age and smoking history were given priority over gender. In 37 LC patients, the exact smoking history was unknown, and in a further four patients, age matching was >3 years.

The group 2 centre also supplied 88 unmatched samples from individuals who were either thought to be normal (n = 25) or have a range of benign lung diseases (n = 63), including mass/nodule (n = 3), autoimmune lung disease (n = 10), chronic obstructive pulmonary disease/emphysema (n = 2), benign pleural effusion (n = 2), allergic/inflammatory/infective conditions (n = 25) (e.g. allergic alveolitis, Wegner’s granulomatosis, asthma, sarcoid, vasculitis, Dessler’s syndrome, mycoplasmosis, tuberculosis) and nonspecified lung disorders (n = 21). A set of individually matched controls for this group of LC patients was selected from a prospective collection of blood samples taken from a normal population in the UK. Controls were matched on the basis of gender and age. With the exception of one patient who was matched to ±4 years, controls were matched to patient age ±2 years. Smoking history was not known for the patients with LC, so controls were simply selected from a population of smokers and ex-smokers. In group 3, there were 199 males and 70 females in the LC group and 187 males and 82 females in the control group. The median age (range) in the LC and control groups was 65 (38–87) and 65 (38–86) years, respectively. The matched controls in group 3 were collected as part of a larger sample set of the normal population (n = 766) in the Midwest United States and demographic data included ethnicity. Evaluation of calibrated reference unit (RU) for autoantibody expression demonstrated that when controlled for age, there was no significant difference between ethnic groups (Caucasians (n = 614), African Americans (n = 108), Hispanics (n = 27) and Native Americans (n = 17)) in terms of calibrated RUs (data not shown). There was a further set of samples from 125 normal individuals who were located in Florida and age matched, gender matched and smoking history matched to a similar number of the controls in the Midwest United States (n = 125). The Florida samples were part of another larger prospective collection of sera from the normal population. Comparison of the 125 samples from each of these two normal populations from different geographic and ethnic backgrounds showed no significant difference in the calibrated RU values for any of the six antigens (data not shown).
Signal stratification of autoantibody levels in serum samples and its application to the early detection of lung cancer

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ABSTRACT

Background: Further signal stratification for the EarlyCDT®-Lung test should facilitate interpretation of the test, leading to more precise interventions for particular patients.

Methods: Samples were measured for the presence of autoantibodies to seven tumor-associated antigens (TAAs) (p53, NY-ESO-1, CAGE, GB4-S, SOX2, MAGE A4, and HuD). In addition to the current test cut-offs (determined using a previously reported Validation case-control sample set, set A; n=501), new high and low cut-offs were set in order to maximize the test’s positive and negative predictive values (PPV and NPV, respectively). All three sets of cut-offs were applied to two confirmatory datasets: (I) the case-control set B (n=751), and (II) Population-derived set C (n=883), and all three datasets combined (n=2,135).

Results: For the Validation dataset, cancer/non-cancer positivity for current cut-offs was 41%/9% (PPV =0.109, 1 in 9). The high positive stratum improved this to 25%/2% (PPV =0.274, 1 in 4). The low negative stratum improved this to 8%/23% (NPV =0.990, 1 in 105). This provides a 25-fold difference in lung cancer probability between the highest and lowest groups.

The test performs equally well in subjects who fulfilled the entry risk criteria for the National Lung Screening Trial (NLST) and subjects who did not meet the NLST criteria.

Conclusions: The EarlyCDT®-Lung test has been converted to a four-stratum test by the addition of high and low sets of cut-offs: patients are thus stratified into four risk categories. This stratification will enable personalization of subsequent screening and treatment programs for high risk individuals or patients with lung nodules.

KEYWORDS

Lung cancer; autoantibody (AAb); tumor-associated antigen; risk stratification

The technical and clinical validation of an autoantibody (AAb) assay for the early detection of lung cancer (EarlyCDT®-Lung) has recently been described (1-3). In a series of case-control studies, where the cases were newly diagnosed lung cancer patients, circulating AAbs to a panel of tumor-associated antigens (TAAs) were measured in serum samples. Validation of the 7 AAb panel showed that EarlyCDT®-Lung can, with a specificity of 93%, detect elevated levels of AAbs in peripheral blood samples for up to 41% of all primary lung cancers (3). In combination with imaging techniques, the test is now commercially available to assist clinicians in the early detection of lung cancer in a high-risk population.

Currently a single test threshold (“cut-off”) for each AAb measured in the panel classifies the samples into two strata, i.e., positive or negative for AAbs associated with lung cancer. This two-stratum test yields a useful binary classification, but given the range of intervention options available to the clinician, refinement of the result is desirable. A four-stratum test is therefore now proposed with additional sets of low and high cut-offs to classify the results into high positive, positive, negative and low negative strata indicating relatively very high, high, low and very low levels of AAbs, respectively; the level of AAbs measured relates to the probability of lung cancer (i.e.,
risk of having the disease) (Table 1). This allows more refined intervention for different sub-groups of patients.

**Materials and methods**

**Assay procedure**

AAbs to seven TAAs (p53, NY-ESO-1, CAGE, GBU4-5, SOX2, MAGE A4, and HuD) were measured using EarlyCDT®-Lung (Oncimmune USA LLC, De Soto, KS, USA), a commercially available blood test based on indirect enzyme-linked immunosorbent assay (ELISA) methods, that uses microtiter plates coated with semi-log serial dilutions of recombinant antigens (1). AAb levels were measured as optical density units, background-corrected and then converted to calibrated reference units (RU). Each patient serum sample was assayed in duplicate on each plate and a titration curve obtained for each antigen. A sample was declared positive if there was a clear titration curve, and if the RU at either of the two highest points on the titration curve was above its respective cut-off for at least one antigen. Quality control samples were interspersed in the sample order.

**Patient samples**

Three separate sets of serum samples were used in this work, two case-control sets described previously, and a new population-based set. All patients provided written informed consent for their samples to be used in this study.

**Sample set A (Validation case-control)**

This set comprised 235 patients with lung cancer from UK, US, Ukraine, and Russia (obtained at or just after diagnosis) representing 87% of the cancers in a previously published dataset (Group 3, n=269) (2) for which enough volume was available to complete the panel of seven AAbs (3). There were 179 non-small-cell lung cancers (NSCLCs, 76%), 53 small-cell lung cancers (SCLCs, 23%), and three others (1%). The controls, all recruited in the US from the general population, came from the same sample set and comprised 266 healthy volunteers with no history of cancer, 235 of whom were matched to the cases by age, gender, and smoking history (2).

**Sample set B (Post-validation case-control)**

Four groups of patients (Groups 1-4) with newly diagnosed lung cancer, but prior to treatment, plus controls matched by gender, age (±4 years) and smoking history (as far as possible), were combined into a single dataset, as previously reported (4). Group 1 comprised 32 cases with SCLC from a single UK center and Group 2 comprised 161 cases from multiple European centers. Controls (±4 years) came from a prospective collection of cancer-free smokers in the Midlands of England and the Midwest of America. Group 3 comprised 120 cases from a single center in Vancouver, Canada, matched to 113 high-risk lung-cancer-free controls. Group 4 comprised 23 cases matched to 109 controls. The total sample set comprised 336 lung cancer cases, including 301 NSCLC (90%) and 35 SCLC (10%), and 415 normal control sera. The incomplete matching in Groups 2 to 4 was mainly due to controls being excluded if they had been used for another group or if sample volume was insufficient.

**Sample set C (Population)**

This set comprised 847 commercially-derived samples collected consecutively between November 2010 and February 2012 from individuals deemed by their clinicians as being at high risk of developing lung cancer. Clinical follow-up information available through a prospective audit is known for all these individuals of whom 36/847 (4.3%) were diagnosed [using computed tomography (CT) and/or biopsy] with lung cancer within 6 months after taking the test. Ethnicity was known for 823 (97%) of patients.

**Derivation of cut-offs**

The current test cut-offs divide the samples into two strata, positive or negative, corresponding to high and low lung cancer risk respectively, so as to maximize the sensitivity for a specificity of about 90% (2). As previously reported (2,3), the specificity was also adjusted for the presence of an estimated small number of undiagnosed cancers in the control group. In the Population dataset, individuals were defined as ‘cancer-free’ if a lung cancer diagnosis was not obtained within six months after testing (manuscript in preparation).

Using sample set A, a new set of high cut-offs, splitting the two-stratum positives, was derived by adding a multiple of

<table>
<thead>
<tr>
<th>Block</th>
<th>Rule</th>
<th>Result</th>
<th>Risk*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>At least one AAb &gt; H</td>
<td>High positive</td>
<td>Very high</td>
</tr>
<tr>
<td>2</td>
<td>All AAbs &lt; H, but at least one &gt; C</td>
<td>Positive</td>
<td>High</td>
</tr>
<tr>
<td>3</td>
<td>All AAbs &lt; C, but at least one &gt; L</td>
<td>Negative</td>
<td>Low</td>
</tr>
<tr>
<td>4</td>
<td>All AAbs &lt; L</td>
<td>Low negative</td>
<td>Very low</td>
</tr>
</tbody>
</table>

AAb, autoantibody; L, low cut-off; C, current cut-off; H, high cut-off; *, Risk (i.e., probability) of having a lung cancer at the time of the test.
the standard deviation of the distribution of controls to the current cut-off for each autoantibody to optimize specificity and sensitivity to yield a high positive predictive value (PPV). Similarly, a set of low cut-offs, splitting the two-stratum negatives, was derived by subtracting multiples of standard deviations to yield a high negative predictive value (NPV). The main calculations were performed assuming a cancer prevalence of 2.7% (2), but tabulation for 4%, being the typical five-year lung cancer risk for an average smoker, was also carried out. All analyses were carried out using SAS® (Version 9.1.3, Cary, NC, USA).

Statistical analysis

The new cut-offs were applied to all datasets, separately and combined, thus sorting patients into four strata on the basis of their EarlyCDT®-Lung AAb levels (Table 1). To check the consistency of the classification, the percentages of samples within the new strata were compared across datasets for cases and controls separately using Fisher Exact tests (5).

Using the specificity and sensitivity for each stratum, the PPV and NPV were then derived using the number of samples in the stratum versus their complement, the samples not in the stratum. A continuous estimate of five-year demographic risk based on gender, age and, where available, smoking history was also derived using a modified version of the Spitz model (6). The demographic factors and staging on the stratification were investigated using multinomial modelling (SAS®, Proc GENMOD and Proc FREQ).

A further analysis compared subjects who could or could not be classified according to the main National Lung Screening Trial (NLST) trial inclusion criteria, i.e., age (55-74 years old) and smoking history (≥30 pack years and quit <15 years ago) (7). In the combined dataset (n=1,802), 531 subjects met the NLST criteria (29%) 116 of whom (22%) were lung cancers, while 1,271 did not (non-NLST) (71%) 328 of whom (26%) were lung cancers (Tables 2, 3). There were more underage subjects than over-age for sets A & B: 21% under-age, 68% NLST and 13% over-age. For sample set C (population set), the figures were 31%, 58% and 11%, respectively. Also, Non-NLST subjects had all smoked less. Two associations were tested (χ² tests): (I) between cancer status and NLST eligibility for each separate test stratum, and (II) between EarlyCDT-Lung positivity and NLST eligibility for cancers and non-cancer subjects separately.

Results

Patient samples

The patient demographics were summarized for the sample sets A, B & C separately (Table 4, full details in Table 5). Demographics for sample sets A & B were representative of patients with lung cancer, with more males than females, age ranging from 23 to 90 years and more than half of patients being at least 60 years old. In sample set C (the Population dataset), however, there was a higher percentage of females, suggesting that females are more likely to be proactive about their health, and with a median age of the cases about 10 years older than for the controls. The pattern of smoking was similar over all three datasets, although with a tendency for the cases to be current smokers and controls to be ex-smokers. Mean demographic risk in sample set C was higher for cases than for controls, reflecting the differences noted above.

Analysis using the current and newly defined test cut-offs

For both the current two-stratum test and the new four-stratum...
test, using the cut-offs derived from sample set A, the sensitivity, specificity, PPV and NPV were calculated for each dataset and for all datasets combined (Table 6, full details in Table 7). For convenience the NPV is also presented in its reciprocal form (1 in X), i.e., the probability of cancer given a negative result.

**Sample set A**

The two-stratum test gave a cancer/normal positivity of 41%/9% (PPV =0.109, 1 in 9), and the four-stratum test high positive stratum improved this to 25%/2% (PPV =0.274, 1 in 4) (Tables 6,7). The two-stratum test also gave a cancer/normal negativity of 59%/91% (NPV =0.982, 1 in 57), and the four-stratum test low negative stratum improved this to 8%/23% (NPV =0.990, 1 in 105). For the demographic split, no difference (5% level) between strata was seen for gender (P=0.99), age category (P=0.053) or smoking status (P=0.37), similarly for staging profile (P=0.16).

**Sample set B**

The two-stratum test gave a cancer/normal positivity of 30%/10% (PPV =0.076, 1 in 13), and the high positive stratum improved this to 17%/4% (PPV =0.113, 1 in 9). The two-stratum test also gave a cancer/normal negativity of 70%/90% (NPV =0.979, 1 in 47), and the low negative stratum improved this to 12%/22% (NPV =0.985, 1 in 65) (Table 6). No difference between strata was seen for gender (P=0.88), age category (P=0.62) or smoking status (P=0.57), similarly for the proportion of Caucasians (P=0.22). There were too few cancers to investigate staging.

**Sample set C**

The two-stratum test gave a cancer/normal positivity of 36%/9% (PPV =0.103, 1 in 10), and the high positive stratum improved this to 19%/2% (PPV =0.226, 1 in 4). The two-stratum test also gave a cancer/normal negativity of 64%/91% (NPV =0.981, 1 in 53), and the low negative stratum improved this slightly to 17%/31% (NPV =0.985, 1 in 67) (Table 6). Again, no difference between strata was seen for gender (P=0.20), age category (P=0.07) or smoking status (P=0.51), similarly for the proportion of Caucasians (P=0.22). There were too few cancers to investigate staging.

**Combined set**

The three datasets were pooled into a single Combined dataset, with 607 cases and 1,492 controls. The two-stratum test gave a cancer/normal positivity of 34%/9% (PPV =0.094,
Table 5. Summary of demographics by dataset.

<table>
<thead>
<tr>
<th></th>
<th>Sample set A dataset</th>
<th>Sample set B dataset</th>
<th>Sample set C (Population) dataset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls N=266</td>
<td>Cases N=235</td>
<td>Controls N=415</td>
</tr>
<tr>
<td>Tumor type, n (%)</td>
<td>NSCLC</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>179 (76%)</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Stage I</td>
<td>79 (34%)</td>
<td>170 (51%)</td>
</tr>
<tr>
<td></td>
<td>Stage II</td>
<td>48 (20%)</td>
<td>45 (13%)</td>
</tr>
<tr>
<td></td>
<td>Stage III</td>
<td>14 (6%)</td>
<td>44 (13%)</td>
</tr>
<tr>
<td></td>
<td>Stage IV</td>
<td>3 (1%)</td>
<td>21 (6%)</td>
</tr>
<tr>
<td></td>
<td>Stage unknown</td>
<td>35 (15%)</td>
<td>21 (6%)</td>
</tr>
<tr>
<td>SCLC</td>
<td>n/a</td>
<td>53 (23%)</td>
<td>n/a</td>
</tr>
<tr>
<td>Limited SCLC</td>
<td>23 (10%)</td>
<td>6 (2%)</td>
<td>n/a</td>
</tr>
<tr>
<td>Extensive SCLC</td>
<td>7 (3%)</td>
<td>26 (8%)</td>
<td>n/a</td>
</tr>
<tr>
<td>Stage unknown</td>
<td>23 (10%)</td>
<td>3 (1%)</td>
<td>n/a</td>
</tr>
<tr>
<td>Type unknown</td>
<td>n/a</td>
<td>3 (1%)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

| Gender, n (%)    | Male                 | 185 (70%)            | 171 (73%)                        | 265 (64%)*                        | 218 (65%)                         | 290 (36%)                         | 15 (42%)                          |
|                  | Female               | 81 (30%)             | 64 (27%)                         | 148 (36%)                         | 118 (35%)                         | 521 (64%)                         | 21 (58%)                          |
| Age, mean +/- sem| 64 +/- 0.6           | 65 +/- 0.6           | 62 +/- 0.5                       | 65 +/- 0.6                        | 61 +/- 0.4                        | 70 +/- 1.4                        |
| Race, n (% of known) | Caucasian           | n/r                  | n/r                              | n/r                              | 721 (91%, n=789)                  | 29 (85%, n=34)                    |
|                  | African-American    | n/r                  | n/r                              | n/r                              | 37 (5%, n=789)                    | 4 (12%, n=34)                     |
| Smoker           | Yes, n (%)          | 93 (35%)             | 108 (46%)                        | 78 (19%)                         | 175 (52%)                         | 361 (45%)                         | 18 (50%)                          |
|                  | No, n (%)           | 36 +/- 2 (n=92)      | 31 +/- 2 (n=86)                  | 31 +/- 3 (n=69)                  | 32 +/- 2 (n=147)                  | 41 +/- 1 (n=346)                  | 45 +/- 6 (n=18)                   |
| Risk (Modified Spitz) | 3.0 (0.3)          | 3.3 (0.3)            | 3.1 (0.3)                        | 3.4 (0.2)                        | 2.4 (0.1)                         | 5.9 (0.6)                         |
|                   | Ex, n (%)           | 144 (54%)            | 67 (29%)                         | 237 (57%)                        | 112 (33%)                         | 331 (41%)                         | 16 (44%)                          |
|                  | No, n (%)           | 32 +/- 3 (n=105)     | 38 +/- 4 (n=37)                  | 31 +/- 2 (n=223)                 | 39 +/- 2 (n=72)                   | 40 +/- 2 (n=315)                  | 52 +/- 9 (n=16)                   |
| Risk (Modified Spitz) | 3.8 (0.2)          | 3.7 (0.3)            | 3.4 (0.1)                        | 3.5 (0.3)                        | 3.7 (0.1)                         | 5.8 (0.4)                         |
|                   | Unknown             | 0 (0%)               | 36 (15%)                         | 1 (0%)                           | 6 (2%)                            | 2 (0%)                            | 0 (0%)                            |

aGender unknown for two subjects. n/a, not applicable; n/r, information not recorded; NSCLC, non-small-cell lung carcinoma; SCLC, small-cell lung carcinoma; sem, standard error of the mean; Pk-yrs, Pack-years. Rounding applied to percentages to ensure 100% totals.

Table 6. Summary of PPV and NPV for two-stratum and four-stratum test.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Two-stratum test</th>
<th>Four-stratum test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive PPV</td>
<td>Negative NPV</td>
</tr>
<tr>
<td>Set A</td>
<td>10.9% (1 in 9)</td>
<td>1.8% (1 in 57)</td>
</tr>
<tr>
<td>Set B</td>
<td>7.6% (1 in 13)</td>
<td>2.1% (1 in 47)</td>
</tr>
<tr>
<td>Set C</td>
<td>10.3% (1 in 10)</td>
<td>1.9% (1 in 53)</td>
</tr>
<tr>
<td>Combined</td>
<td>9.4% (1 in 11)</td>
<td>2.0% (1 in 51)</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value, in its reciprocal form, i.e., 1-NPV. Based on a population lung cancer prevalence of 2.7%.
1 in 11) and the high positive stratum improved this to 20%/2% (PPV =0.193, 1 in 5). The two-stratum cut-offs also gave a cancer/normal negativity of 66%/91% (NPV =0.980, 1 in 51) and the low negative stratum improved this to 11%/27% (NPV =0.989, 1 in 90) (Table 6). Further analysis showed clear consistency of the EarlyCDT-Lung risk profile across age decades (Tables 8,9).

### Comparison across datasets

The positivity percentages were consistent across the three datasets for both cases and controls (Table 6). For the low negative stratum, the percentage of negatives in sample set C ‘controls’ (31%) was higher than for the other two datasets (23% and 22% respectively, P=0.001), which could reflect the higher number of younger cancer-free individuals in the population sample set C. Even despite the age difference, some dataset-to-dataset variation is to be expected, and the difference was not great. This consistency confirmed that the new sample set A cut-offs were directly applicable to sample sets B & C.

### Risk analysis

In standard demographic models (e.g., Spitz) (6), risk increases with age and degree of smoking. To assess the independence of demographic risk and EarlyCDT-Lung result, a single threshold was applied to demographic risk to classify samples into low and high risk. This allowed 2×2×2 tables of positivity (demographic risk, EarlyCDT-Lung result, cancer status) to be compiled, bearing in mind the matching in the case-control sets. No evidence was found for a departure from independence (proportionality) of demographic risk and EarlyCDT-Lung.

The modification of the personalized continuous demographic risk by the four-stratum test is also under investigation. Based on DLR (diagnostic likelihood ratio) calculations (8) for typical cases (e.g., middle-aged moderate smokers), going from a positive result in the two-stratum test to a high positive result in the four-stratum test changed the risk increase from 4.3- to 12.7-fold. Similarly, going from a two-stratum negative result to a four-stratum low negative result changed the risk decrease from 1.5- to 2.9-fold.
**EarlyCDT-Lung and NLST criteria**

There was no evidence that the cancer rate differed between NLST and Non-NLST cohorts at the positive end of the test (Table 2). At the negative end, there were some small differences, but these were not consistent across sample sets A & B (more cancers in the Non-NLST cohort) and sample set C (Population dataset) (more cancers in the NLST cohort). Statistical significance was generally only seen when the table frequencies were high, and in fact, the differences were not large in the NPV estimates.

There was also little evidence for a difference between NLST and Non-NLST cohorts in their positivity profile. The only comparison significant at 5% was for the sample sets A & B cancers in the four-stratum test where there were more high positives in the NLST cohort (30%) than in the Non-NLST cohort (18%) (Table 3), but this finding was not repeated in sample set C (Population dataset).

**Table 8. Age by EarlyCDT-Lung risk stratum, combined dataset (Cancers).**

<table>
<thead>
<tr>
<th>Stratum</th>
<th>Age group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20-49</td>
<td>50-59</td>
</tr>
<tr>
<td>Very low risk</td>
<td>2 (5%)</td>
<td>16 (12%)</td>
</tr>
<tr>
<td>Low risk</td>
<td>34 (78%)</td>
<td>74 (53%)</td>
</tr>
<tr>
<td>High risk</td>
<td>2 (5%)</td>
<td>18 (13%)</td>
</tr>
<tr>
<td>Very high risk</td>
<td>5 (12%)</td>
<td>31 (22%)</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>139</td>
</tr>
</tbody>
</table>

**Table 9. Age by EarlyCDT-Lung risk stratum, combined dataset (Controls).**

<table>
<thead>
<tr>
<th>Stratum</th>
<th>Age group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20-49</td>
<td>50-59</td>
</tr>
<tr>
<td>Very low risk</td>
<td>58 (33%)</td>
<td>139 (30%)</td>
</tr>
<tr>
<td>Low risk</td>
<td>98 (57%)</td>
<td>281 (60%)</td>
</tr>
<tr>
<td>High risk</td>
<td>14 (8%)</td>
<td>38 (8%)</td>
</tr>
<tr>
<td>Very high risk</td>
<td>4 (2%)</td>
<td>8 (2%)</td>
</tr>
<tr>
<td>Total</td>
<td>174</td>
<td>466</td>
</tr>
</tbody>
</table>

Percentages calculated within column. Test for an association between stratum profile within age.

**Discussion**

Improvements in diagnostic test sensitivity and specificity, and hence PPV and NPV, facilitate clinical intervention decisions. This report confirms that the addition of high and low cut-offs to EarlyCDT-Lung enables stratification of patients into very high risk for lung cancer, with improved PPV, or very low risk, with improved NPV.

Three lung cancer case-control sets were assessed. The case demographics were representative of patients with lung cancer: a predominance of males, more than half of patients >60 years of age, and over half the patients with early-stage lung cancer (i.e., NSCLC stages 1 or 2 or limited SCLC).

For the high positive stratum the specificity was set at 98%. In sample set A, this lowered the sensitivity from 41% for the positive stratum to 25%, but overall the PPV was greatly increased from 10.9% (1 in 9) to 27.4% (1 in 4). Similarly, for the low negative stratum the NPV increased from 98.2% (1 in 57) to 99.0% (1 in 105). The cost for this improvement is reduced performance for the two intermediate strata; for the positive stratum the PPV fell to 5.5% (1 in 18), whilst for the negative stratum the NPV fell to 98.0% (1 in 49).

Importantly, the consistency of performance when applied to sample sets B and C was found to be excellent (Tables 6,7). These data suggest that the EarlyCDT-Lung measurements may provide a continuous variable in terms of lung cancer risk. We term this the Occurrence Score and it is under development.

There was no evidence for an association between demographic factors and EarlyCDT-Lung strata. The analysis clearly suggested that EarlyCDT-Lung is adding to demographic risk independently.

The varied origin of the sample sets supports the general applicability of the results. Nodule data was not available for the case-control datasets. In the Population dataset, however, a positive EarlyCDT-Lung result did add to the risk of a lung nodule being cancer (manuscript in preparation). The described AAb technology and CT imaging are potentially additive...
rather than competitive since the presence of AAbs provides an opportunity for early detection of lung cancer, even in early-stage disease, and may therefore be useful in the management of high-risk individuals. Thus, for example, combining a low negative EarlyCDT®-Lung result with a negative CT scan would lead to a very high NPV (manuscript in preparation).

Finally, this study compared the EarlyCDT-Lung strata with whether or not patients met the entry criteria for the NLST study. Only 65% of participants in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO) who developed lung cancers met the NLST criteria (9), and in another recent US study of early stage lung cancer patients (n=267) fewer than half met the criteria and would not be covered under current screening paradigms (10). In our analysis of EarlyCDT-Lung, little evidence was found that the cancer rate differed between NLST and Non-NLST cohorts, indicating that EarlyCDT-Lung provides similar risk stratification for these cohorts. Thus we can now identify individuals initially deemed at a risk lower than the NLST criteria whose risk after EarlyCDT-Lung is equivalent to the entry criteria for the NLST. This provides a rationale for identification and CT screening of individuals who fall outside the NLST criteria.

**Conclusions**

EarlyCDT-Lung is recommended as a tool for physicians to assess a patient’s probability of lung cancer thereby facilitating the early detection of lung cancer. By applying two additional cut-offs, we have converted the test to a four-stratum version to allow further stratification of patients into different risk categories. This enhanced stratification can be used on a population that fulfills the NLST criteria to identify super high risk sub-groups. In addition, we have shown that EarlyCDT-Lung can increase the risk estimates for certain Non-NLST patients, and bring them into the NLST range, thus facilitating more appropriate intervention for such patients.

**Acknowledgements**

Funding: This work was supported by the University of Nottingham and Oncimmune Ltd.

The manuscript was drafted by the authors. However, the authors would like to acknowledge and thank Varinia Munoz, from Complete Medical Communications, who provided editorial assistance funded by Oncimmune Ltd. Thanks also to Jared Allen, Oncimmune Ltd., for data-processing and IT support.

**Disclosure:** JFR is Chief Scientific Officer and a shareholder in Oncimmune Ltd, a University of Nottingham spinout company; G. Hamilton-Fairley is a shareholder and Executive Chairman of Oncimmune Ltd; G. Healey is an employee of Oncimmune Ltd.; L. J. Peek is an employee of Oncimmune LLC. All remaining authors have declared no conflicts of interest.

**References**

EarlyCDT-Lung

NHS 12,000 Patient Study

The study is the largest randomized trial for the early detection of lung cancer using biomarkers ever conducted.
Promising Early Results of 10,000 Patient Lung Cancer Screening Trial Using EarlyCDT(R)-Lung Blood Test Announced at WCLC

TORONTO, Sept. 8, 2015 -- GeneNews Limited ("GeneNews" or the "Company") (TSX:GEN) today received encouraging early results from the largest randomized trial ever conducted for the early detection of lung cancer using EarlyCDT®-Lung, an autoantibody biomarker blood test. The findings were announced earlier this morning at the International Association for the Study of Lung Cancer’s 16th World Conference on Lung Cancer ("WCLC").

The National Health Service ("NHS") Scotland-sponsored Early Cancer Detection Test – Lung Cancer Scotland ("ECLS") Study of 10,000 high-risk smokers demonstrated a cancer detection rate (sensitivity) of 81% for EarlyCDT®-Lung in these initial results. While the control arm in the study has not been formally assessed, the positivity rate was as expected with a specificity of 91%. The final data on the control arm will be collected at the end of the study.

EarlyCDT®-Lung is a blood test, easily done, that aids physicians in risk assessment and the early detection of lung cancer in high-risk, asymptomatic patients. GeneNews’ Innovative Diagnostics Laboratory LLP ("IDL") joint-venture is licensed to distribute and perform the EarlyCDT®-Lung test in the United States. The test is reimbursed by both Medicare and commercial insurers.

First announced in March 2012, the ECLS Study was established to determine if the use of EarlyCDT®-Lung leads to earlier detection of lung cancer and can help to save lives in the long term. As part of the study, half of the patients pre-identified as high-risk for lung cancer were followed up by usual care, and half were asked to take the EarlyCDT®-Lung test. Those who received a positive result were effectively triaged into a much higher risk group and referred for X-ray and low dose computerized tomography (CT) scans. This higher-risk selection protocol results in many fewer low dose CT scans, but with the same mortality benefit of low dose CT scans already established in the U.S. National Lung Screening Trial ("NLST").

"Lung cancer is usually detected only in its late stages when symptoms are evident, treatment more complicated, and survival lower," said James Howard-Tripp, Executive Chairman, GeneNews. "This is why the adoption of EarlyCDT®-Lung as an indicator of early lung cancer is so important. It is an aid to physicians in assessing high-risk patients and then stratifying those with a positive finding to increased testing, with the objective of finding the disease early and treating it early. This is the message we can take to the physicians, patients and insurers with greater clarity now."

"ECLS has recruited nearly 10,000 patients to-date and will complete the recruitment of an additional 2,000 patients in early 2016 with full results available after two years of follow-up," explained Professor Frank Sullivan, Chief Investigator of the ECLS Study, who presented the trial results at WCLC. "It has been a major effort to recruit such an impressive number of study participants."

Further interim results from the study will be announced over the next three years with final publication of the study anticipated in 2018-2019. Once concluded, the trial will enable the NHS to determine whether to offer the EarlyCDT®-Lung blood test as a nationwide screening in the future, and may enable the adoption of the testing by other countries.
About the ECLS Study

Lung cancer kills more people than any other cancer worldwide, with over 1.5 million deaths globally in 2012. In the United States, more than 155,000 people die from lung cancer every year.

It is often hard to find lung cancer early. Most people with early lung cancer do not have any symptoms, so only a small number of lung cancers are found at an early stage when treatment can be most successful. It is for this reason that the Scottish Government is co-funding the ECLS Study along with Oncimmune Ltd, the company who developed EarlyCDT®-Lung, the autoantibody biomarker test being trialed in the study. This EarlyCDT®-Lung blood test may be able to pick up very small lung cancers before symptoms are evident.

The study originally invited 10,000 high-risk people from Scotland's Tayside, Glasgow and the surrounding areas to participate. An additional 2,000 patients will now be recruited and some of these will come from Lanarkshire. These regions have been chosen because lung cancer is more common in these areas. Those who agree to participate in the ECLS Study either receive an EarlyCDT®-Lung test or are followed up by usual care. Patients with a positive blood test are offered a chest X-ray and a series of CT scans over two years. All participants requiring further investigations or treatment are treated within NHS guidelines. For more information visit: http://www.eclsstudy.org/home.

A key outcome of this study will be the cost-effectiveness of screening high-risk patients with EarlyCDT®-Lung. Although the first results will be published in 2018-2019, ECLS Study investigators want to find out if earlier detection saves lives in the long term by following everyone who takes part in the study for up to 10 years. Patient feedback regarding EarlyCDT®-Lung also will be sought as part of the NHS decision process to determine whether the test should be offered as a nationwide lung cancer-screening test.

About EarlyCDT-Lung

EarlyCDT®-Lung is a simple blood test which is ordered by a physician to aid in the risk assessment and early detection of lung cancer in moderate and high risk patients, and to stratify indeterminate pulmonary nodules for the risk of malignancy. The test’s overall accuracy is greater than 91%.

When a tumor is present it produces abnormal proteins (known as antigens). Antigens from a person's own cells are not normally found in the body. The body reacts to these antigens by producing autoantibodies. The test measures a panel of seven autoantibodies to detect the presence of lung cancer.

EarlyCDT®-Lung has been developed so that individuals at moderate or high risk of developing lung cancer can benefit from an increasing chance that lung cancer can be detected at the earliest possible stages, when treatment can be most successful. The EarlyCDT®-Lung test can also be used in conjunction with diagnostic imaging such as X-ray or CT scans to further assess the risk of lung cancer being present where indeterminate lung nodules have been detected which may or may not be a sign of cancer.

About GeneNews

GeneNews is focused on developing and commercializing proprietary molecular diagnostic tests for the early detection of diseases and personalized health management, with a primary focus on cancer-related indications. The Company's lead product, ColonSentry®, is the world's first blood test to assess an individual's current risk for colorectal cancer. In 2013, GeneNews created a U.S. joint venture, Innovative Diagnostic Laboratory, LLP ("IDL"), that it is committed to help become a leader in molecular diagnostics and personalized medicine, serving as a strong commercialization outlet for advanced cancer tests. Taking a multi-view approach to the diagnosis and treatment of cancer, IDL is working to assemble, through a combination of internal pipeline development, third-party licenses and potential acquisitions, a robust menu of novel, proprietary tests to be offered by it throughout the United States. GeneNews' common shares trade on the Toronto Stock Exchange under the symbol 'GEN'. More information on GeneNews and IDL can be found at www.GeneNews.com and www.MyInnovativeLab.com, respectively.
EarlyCDT-Lung

Regulatory Information
CE MARK APPROVAL

On July 15, 2016 Oncimmune announced that it had received a European CE for its product EarlyCDT-lung. The CE mark certifies that the reagents used in the EarlyCDT-lung test meet the European Union’s standards for quality control and manufacturing, to enable the product to be placed on the market.

REGULATORY INFORMATION

Oncimmune’s testing is performed in a registered, high-complexity laboratory. They meet or exceed all CLIA and CAP regulations and are compliant with OSHA regulations (OSHA – Occupational Safety & Health Administration). Following an assessment by BSI, Oncimmune’s Quality Management System is now certified to EN ISO 13485:2012 standard for the design, development and manufacture of in vitro diagnostic immune biomarker devices for early detection of solid tumour cancers (ISO Certificate). Oncimmune® (UK) Ltd complies with the Data Protection Act 1998.
EarlyCDT-Lung

Evaluation & Customer Support
Lung Cancer Clinical Advisory Group

- Professor Peter Boyle
- Professor Herb Fritsche
- Dr. Neal Navani
- Professor Jim Jett MD: Pulmonologist, National Jewish, Denver
- Professor Frank Detterbeck: Head of Thoracic Surgery, Yale University, CT;
- Professor Pierre Massion: Pulmonologist Vanderbilt, Nashville; 29 Professor
- Tim Kennedy: Pulmonologist University of Colorado;
- Professor Peter Mazzone: Pulmonologist Cleveland Clinic.

Evaluation & Customer Support

- More than 120,000 patient samples were run and 12 million data points analyzed to validate the technical and clinical performance of EarlyCDT-Lung.

- More than 145,000 commercial tests have also been run in the US laboratory.

- Currently, EarlyCDT-Lung is being used in the largest randomized trial for the early detection of lung cancer using biomarkers ever conducted; the National Health Service (NHS) Scotland ECLS study of 12,000 high-risk smokers.
Testimonial Video
Click Below to Watch

Dr. Keith Kelley on the Value of Early Detection of Lung Cancer