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Imaging, Diagnosis, Prognosis

Immunobiomarkers in Small Cell Lung Cancer: Potential Early Cancer Signals

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Abstract

Purpose: We investigated the presence of autoantibodies as immunobiomarkers to a panel of tumor-associated antigens in a group of individuals with small cell lung cancer (SCLC), a disease group that has a poor overall cancer prognosis and therefore may benefit most from early diagnosis.

Experimental Design: Sera from 243 patients with confirmed SCLC and normal controls matched for age, sex, and smoking history were analyzed for the presence of these early immunobiomarkers (i.e., autoantibodies to p53, CAGE, NY-ESO-1, GBU4-5, Annexin I, SOX2, and Hu-D) by ELISA.

Results: Autoantibodies were seen to at least 1 of 6 antigens in 55% of all the SCLC patients' sera tested, with a specificity of 90% compared with controls. Using a higher assay cutoff to achieve a specificity of 99%, autoantibodies were still detectable in 42% of SCLC patients (receiver operator characteristic area under the curve = 0.76). There was no significant difference in sensitivity when analyzed by stage of the cancer or by patient age or gender. The frequency of autoantibodies to individual antigens varied, ranging from 4% for GBU4-5 to 35% for SOX2. Levels of Annexin I autoantibodies were not elevated in patients with SCLC. Antibodies were also detected in 4 separate patients whose sera were taken up to 3 months before tumor diagnosis.

Conclusion: The presence of an autoantibody to one or more cancer-associated antigens may provide an important addition to the armamentarium available to the clinician to aid early detection of SCLC in high-risk individuals. *Clin Cancer Res; 17(6); 1474–80. ©2011 AACR.*

Introduction

Lung cancer is the largest cause of death from cancer worldwide, being responsible for more than 1.2 million deaths every year. While tobacco smoking is still the major contributing factor (estimated to cause around 90% for all cases; refs. 1, 2), other recognized risk factors for lung cancer include passive smoking, radon exposure, and occupational exposures, especially to asbestos, arsenic, and polycyclic hydrocarbons (1). It is estimated that the latency period for lung cancers attributable to smoking is at least 20 years (1), yet lung cancer is often only detected at an advanced stage, with little prospect of curative treatment. Presently, there is no accepted early diagnostic test, although screening trials using spiral computed tomography (CT) in at-risk individuals are ongoing (3).

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The incidence of small cell lung cancer (SCLC) has decreased in the United States in recent years, from 17% of all lung tumors in 1986 to 13% in 2002, with equal numbers of men and women now presenting with the disease (4). In other countries, the incidence of SCLC is still reported at closer to 20% (3, 5). At presentation, the vast majority of patients with SCLC are symptomatic (6) and from the date of diagnosis, the average survival time for SCLC patients with limited disease (LD) is approximately 18 months, reduced to only 9 months for those with extensive disease (ED; ref. 7). Untreated, SCLC has the most aggressive clinical course of any type of lung cancer, with a median survival from diagnosis of only 2 to 4 months, thus showing the urgent need for earlier diagnosis (7). SCLC is more responsive to chemotherapy and radiation therapy than other types of lung cancer; however, a cure is difficult to achieve because SCLC has a greater tendency to be widely disseminated by the time of diagnosis.

Historically, surgery has not been commonly used in the treatment of SCLC but new surgical and adjuvant chemotherapy regimens, particularly platinum-based regimens, are beginning to show promise in LD patients (8). A recent review of this subject has highlighted encouraging results in stage I patients with 5-year survival rates of up to 58% overall and 73% in patients with stage IA cancers (9). Lim and colleagues reported a 5-year survival of 52% for

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Translational Relevance

This study shows the clinical relevance of measuring the antibody response to a panel of tumor-associated antigens, by ELISA, in a large prospective cohort of individuals with small cell lung cancer (SCLC) and shows the sensitivity and specificity of such a test when compared with an age-, gender-, and smoking-matched set of individuals. SCLCs comprise a disease group that have a particularly poor overall cancer prognosis, due to their late presentation, and therefore a group that are most at need of early diagnosis. It would be envisaged that this simple, reliable, and noninvasive blood test could aid the early detection of SCLC in a high-risk population and could be most effectively used to direct imaging modalities with low specificity such as spiral computed tomography. Such a test could therefore have a significant impact on the long-term survival of these individuals.

stage I disease without adjuvant therapy (10), which strongly suggests that if found early, SCLC may still be a localized disease. Brock and colleagues reported that in their series of stage I SCLC, the 5-year survival with platinum-based adjuvant chemotherapy was 85.7% (8). These studies indicate a potentially significant survival advantage from both early diagnosis and platinum-based adjuvant chemotherapy. A test that could identify such cancers at an early stage is critical to increase the chance of successful treatment.

There is an increasing body of literature describing the presence of a humoral immune response, in the form of autoantibodies, to tumor-associated antigens (TAA) in lung and other solid tumors (11–17). Autoantibodies have been described as being present in some individuals before developing symptomatic cancer (13, 18–20), making their identification of particular relevance for early detection. We have recently reported the development of a highly reproducible assay to measure the presence of autoantibodies in primarily non-SCLC (NSCLC; ref. 16). We now expand these results by reporting on autoantibody detection in a large prospective cohort of patients with SCLC collected from a single center.

The panel of 7 antigens selected in this study comprises a number of well recognized cancer-associated proteins including 6 antigens (p53, SOX2, Annexin I, CAGE, GBU4-5, and NY-ESO-1) as previously reported (16) along with an additional antigen Hu-D. Autoantibodies to the *p53* tumor-suppressor gene were first described in 1982 (21) and have now been observed in a wide variety of malignancies (14–17). Autoantibodies to SOX2, a member of the SOX-B1 family of genes thought to be important in neurogenesis, have been reported in up to 33% of individuals with SCLC (22–24) and considered to be a specific serologic marker for SCLC (22). Autoantibodies to Annexin I and 3 SEREX-identified proteins, GBU4-5 (TDRD12), CAGE, and NY-ESO-1 have also been reported in lung cancer (15, 16, 25). CAGE and NY-ESO-1 are members of the cancer testis antigen family, whereas annexin I is a calcium- and phospholipid-binding protein that is considered to play an important role in tumorigenesis. Autoantibodies to Hu-D, a member of the family of onconeuronal RNA-binding proteins (also known as neuronal-Embryonic Lethal, Abnormal Vision-like gene), have also been described particularly in patients with SCLC and are classically associated with paraneoplastic neurologic diseases (PND) such as paraneoplastic encephalomyelitis/sensory neuropathy (26). Low titers of anti-Hu antibody have been reported in up to 16% of patients with neuroendocrine tumors such as SCLC, even in the absence of well-defined PND (26, 27). A further antigen, recoverin, was also investigated, as autoantibodies to recoverin are known to be associated with cancer-associated retinopathy (CAR; ref. 6) and have also been reported in patients with NSCLC and SCLC without CAR (28).

This study is the largest of its kind in SCLC to date and, unlike previous reports, includes an equal number of control samples matched for age, gender, and smoking history, thereby minimizing the possibility of overestimating the diagnostic potential of any one autoantibody.

Material and Methods

Blood samples and patient details

Serum samples were collected from 243 consecutive unselected patients, who consented to the study, either with biopsy-proven SCLC or with a characteristic PND if further follow-up investigations revealed SCLC. All patients were seen within the Trent region, UK, between 2005 and 2010 (Nottingham Research Ethics Committee approval 04/Q2404/100). All patients underwent full neurologic evaluation and examination, and serum samples were taken prior to chemotherapy. This sample set comprised more than two thirds of all newly diagnosed SCLC patients in the Trent region during this study period. Two hundred thirty-seven of the samples were obtained at or just after histopathologic confirmation of the tumor, with 1 sample obtained 4 months before an SCLC relapse. Ten of the patients with SCLC also had Lambert–Eaton myasthenic syndrome (LEMS), and 4 of these samples were taken 1 to 3 months before SCLC had been confirmed. Four additional samples were also obtained from individuals considered to be at an increased risk of developing SCLC (1 with LEMS and 3 with a subacute sensory neuronopathy). Another individual, originally thought to be at risk of developing SCLC, was found to have 2 suspicious lung nodules at the time the blood was taken and had her SCLC subsequently confirmed 18 months later.

For antibody analysis, the control group consisted of 247 healthy volunteers recruited in the same region of the United Kingdom who were matched to the SCLC and at-risk patients according to age, gender, and smoking status and had no evidence of any current or prior cancer.

All serum samples were collected and stored at −70°C prior to analysis.

Antigen production

Specific cDNAs for p53, NY-ESO-1, CAGE, Hu-D, SOX2, Annexin I, GBU4-5, and recoverin were subcloned, along with a small tag, into the pET21b expression vector (Novagen) as previously described (15, 16). The recombinant proteins and a negative control protein (tag alone) were expressed, purified, and analyzed as described elsewhere (16, 29).

Autoantibody detection

Autoantibody detection was by ELISA, using microtiter plates coated with set of semilog serial dilutions of recombinant antigens, as previously described (16). All assays were conducted on a semiautomated robotic system. For all assays, samples were measured in duplicate on at least 2 separate occasions. All cancer and normal samples were interspersed, and a calibration system and control samples were also run to allow for quality control monitoring of the assay runs and to correct for any day-to-day variation. A subset of cancer and normal sera were also investigated for the presence of autoantibodies to recoverin; in this case, a calibration system was not used.

Positive seroreactivity was defined as (a) having evidence of a dose response to the antigen titration series (30) and (b) a calibrated optical density (OD) value (RU; of the background corrected signal) above a cutoff level set from the matched control data. Panel sensitivity for the detection of SCLC was defined as the presence of an autoantibody to 1 or more of a panel of 6 antigens (p53, NY-ESO-1, CAGE, Hu-D, SOX2, and GBU4-5). Two cutoffs were applied such that the panel specificity was 99% or 90% specific for cancer detection (mean + 9 SD or 3–4 SD of the normal population for each antigen). Samples were designated positive for each separate autoantibody assay if there was a reproducible signal above the cutoff level. Specificity of the assay was calculated as the percentage of controls that gave a negative result.

Statistics

Standard descriptive statistics such as frequency, mean, and SD were calculated to describe the study population. All analyses were done using Microsoft Excel, SPSS, or GraphPad Prism software. The number and proportion of positive samples were presented with 95% exact CI for binomial proportions (31). Chi-squared tests were used to determine when the proportion of positive results was significantly different between cancer groups and the normal controls.

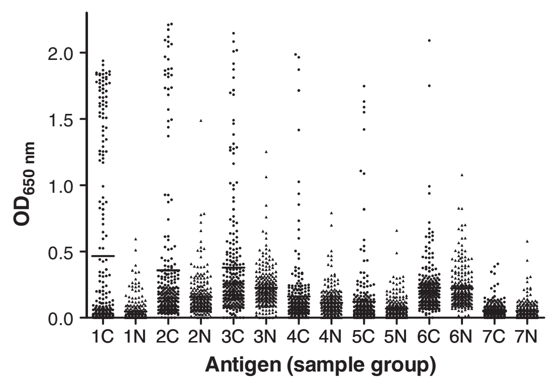

Figure 1. ELISA antibody titers of individual patients and normal controls for TAAs. Scatter plots of OD values of autoantibodies from SCLC sera (242) and matched normal sera (247) to (1) SOX2, (2) Hu-D, (3) p53, (4) NY-ESO-1, (5) CAGE, (6) GBU4-5, (7) Annexin Ia, in cancer (C) and normal (N) sera. Mean values are shown.

Results

Patient and normal control demographics and tumor characteristics are shown in Table 1.

Where complete data on the smoking history of the normal individuals were available ($n = 189$), the estimated risk of developing a lung cancer within the next 5 years was calculated (according to the risk model proposed by Spitz and colleagues, ref. 32) to be 2.16%, ranging from 0.1% to 9.8%, showing the appropriateness of the control group selected.

The presence of autoantibodies to 7 of the TAAs is shown for 1 concentration of antigen in the scatter plots in Figure 1 and clearly shows that elevated levels of autoantibodies (when compared with matched control sera) are present in individuals with SCLC for 6 of the antigens investigated.

Table 1. Patient details and clinical characteristics

	SCLC sera			At risk	Normal control sera
	All	LD	ED		
Group, *n*	243	90	153	4	247
Mean age	66	66	66	69	66
SD	9.6	10.4	9.1	15.5	9.6
Age range	33–87	33–87	43–86	55–87	33–87
Female, *n* (%)	118 (49)	49 (52)	69 (47)	2 (50)	121 (49)
% Smokers/ex-smokers	99	99	99	75	99

NOTE: LD cohort consist of individuals with stage III ($n = 61$), stage II ($n = 15$), and stage I ($n = 14$, of which 5 with stage IA) disease.

Table 2. Frequency of autoantibodies to TAAs

Group	SOX2	Hu-D	p53	NY-ESO-1	CAGE	GBU 4-5	Panel
A. Individual autoantibody positivity derived using cutoffs leading to overall panel specificity of 90%							
All SCLCs	35 (29–41)[a]	13 (9–18)[a]	16 (12–21)[a]	6 (3–10)[b]	7 (4–11)[c]	4 (2–7)[c]	55 (48–61)[a]
LD	36 (26–46)[a]	17 (10–26)[a]	17 (10–26)[a]	4 (1–11)[b]	3 (1–9)[b]	3 (1–9)[b]	53 (43–64)[a]
ED	34 (27–42)[a]	11 (7–17)[a]	16 (10–22)[a]	7 (4–11)[b]	9 (5–15)[d]	5 (2–9)[c]	56 (47–64)[a]
Matched normals	3 (1–6)	1 (0–4)	2 (1–5)	3 (1–6)	2 (1–5)	1 (0–4)	10 (7–15)
Specificity	97	99	98	97	98	99	90
B. Individual autoantibody positivity derived using cutoffs leading to overall panel specificity of 99%							
All SCLC	29 (23–35)[a]	9 (6–14)[a]	12 (8–16)[a]	3 (1–6)[c]	4 (2–7)[d]	1 (0–3)[b]	42 (36–48)[a]
LD	29 (20–39)[a]	10 (5–18)[a]	13 (7–22)[a]	3 (1–9)[b]	0 (0–4)	1 (0–6)[b]	41 (31–52)[a]
ED	29 (22–37)[a]	9 (5–15)[a]	10 (6–16)[a]	3 (1–7)[c]	6 (3–11)[d]	1 (0–4)[b]	42 (35–51)[a]
Matched normals	0 (0–1)	0 (0–1)	0 (0–2)	1 (0–3)	0 (0–1)	0 (0–1)	1 (0–3)
Specificity	100	100	>99	99	100	100	99

NOTE: All values are given in percentage positivity with 95% CI in each patient group. Panel: autoantibody positivity to any 1 of the 6 antigens. *P* value is relative to normal controls (χ^2 analysis).
[a]*P* < 0.001.
[b]*P* value not significant > 0.05
[c]*P* < 0.05.
[d]*P* < 0.01.

There was no difference in signal between the cancer and normal data sets for the presence of autoantibodies to the Annexin I antigen (2% sensitivity, 98% specificity). The antigen was also not additive in terms of improving overall sensitivity and specificity of the panel and so was not included in the final panel analyses.

A subset of samples was also analyzed for the presence of antibodies to recoverin. Autoantibodies were detected in only 4% of the SCLC subset tested. These autoantibodies were detected in samples that already had raised levels of autoantibodies to 1 of the other 6 TAAs; therefore, recoverin was also excluded from the full panel.

The level and relative importance of autoantibody responses to individual antigens in the panel assay varied (Table 2). Table 2 also shows levels of detection of autoantibodies against individual antigens in the LD and ED groups and normal controls. Individual assay sensitivity ranged from 4% to 35%, with specificity for each antigen (for all normal sera) being 97% to 99% (Table 2, A). Autoantibodies to all the antigens could be detected at similar frequencies in both the LD and ED cohorts apart from the autoantibodies to CAGE which seem to be more associated with the presence of ED.

The sensitivity of the panel assay to correctly identify SCLC is shown in Table 2 and graphically represented by the receiver operator characteristic (ROC) curve in Figure 2. Panel sensitivity for the detection of SCLC was 55% (51% LD, 57% ED), with specificity for cancer detection at 90% (Table 2, A). An alternative 6-antigen panel, which included Annexin I but not Hu-D, gave a sensitivity of 51% (47% LD, 53% ED), with a specificity of 90%.

There were no significant differences in sensitivity of the autoantibody panel to detect different stages of disease, with similar levels of sensitivity seen across all the stages from stage IA to those with extensive (metastatic) disease ($P = 0.41$; Fig. 3). The level of sensitivity was also independent of lymph node involvement ($P = 0.61$).

Nine of 10 patients with SCLC and LEMS were positive in the autoantibody assay, with 8 having high titers of antibodies to the SOX2 antigen. Removal of these

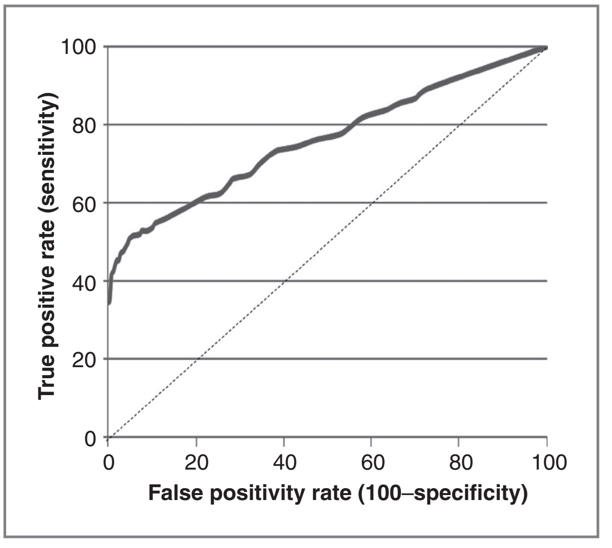

Figure 2. ROC of the panel for the detection of SCLC. ROC area under the curve (AUC) = 0.761 (se 0.027) for the panel. Individual antigen AUC: SOX2, 0.662 (se 0.031); Hu-D, 0.598 (se 0.032); p53, 0.588 (se 0.032); NY-ESO-1, 0.515 (se 0.032); CAGE, 0.517 (se 0.032); GBU4-5, 0.500 (se 0.032).

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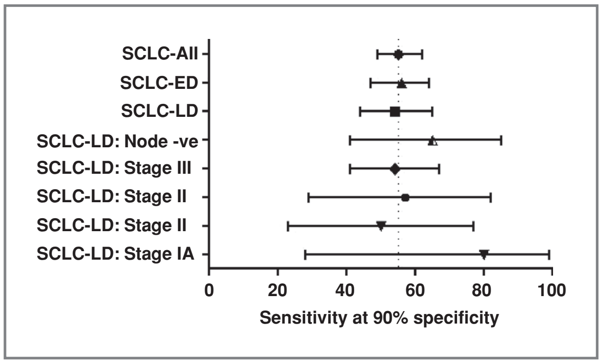

Figure 3. Forest plot showing the sensitivity at a fixed specificity of 90% by tumor stage and nodal status. Line shows sensitivity of 55%. Node negative ($n = 20$, stages I and IIA).

particularly high-risk individuals from the analysis did not significantly decrease the sensitivity of the panel for the detection of cancer (53%).

Restriction of the panel to the presence of SOX2, Hu-D, and p53 autoantibodies correctly identified 48% of SCLCs, with specificity for cancer detection of 94%. There was again no statistical difference between the sensitivity for ED (48%; 95% CI: 40%–57%) and LD (46%; 95% CI: 36%–57%; $P = 0.72$).

Within the LD cohort, 14 patients were confirmed to have stage I disease (5 being stage IA) and, although numbers were small, autoantibodies to p53, SOX2, or Hu-D were detected in 80% of stage IA and 50% of the stage I cancers overall. Notably, 4 of these stage I individuals had samples taken between 1 and 3 months before first SCLC diagnosis, due to detection of a PND, and all 4 had autoantibodies to SOX2. The individual who had a sample taken 4 months before SCLC relapse (again with PND) also had autoantibodies to SOX2.

Three of the 5 of patients who had never smoked had elevated levels of autoantibodies, and the 4 individuals considered to be at a high risk of developing SCLC (due to their PND) also had raised levels of autoantibodies to 1 of the 6 antigens.

An increased specificity for detecting SCLC could be achieved by increasing the cutoff (for cancer detection) by using a mean + 9 SD of the normal population (Table 2, B). Under these conditions, 99% of the normal samples and 42% of the SCLC samples were correctly identified, again with no statistical difference between the detection of LD or ED types ($P = 0.32$), different stages of disease ($P = 0.76$), or nodal status ($P = 0.86$). All 4 of the prediagnostic samples were still identified at this level of specificity, as well as 2 of the 4 at-risk individuals. The limited panel of p53, SOX2, and Hu-D identified 37% of the SCLC samples at greater than 99% specificity.

Discussion

Autoantibodies to TAAs are revealing themselves as important immunologic biomarkers for the (early) detection of cancer. More than half of the SCLC samples tested here had autoantibodies to at least 1 of 6 TAAs, with the majority of these samples having high titers of autoantibodies to 1 or more of SOX2, p53, and Hu-D, antigens highly associated with SCLC. Autoantibodies to Annexin I were also investigated in all the samples tested, having previously been included in a panel to detect primary lung cancer (both NSCLC and SCLC; ref. 16).

SCLC is also the cancer most commonly associated with PNDs which occurs in up to 6% of patients with this tumor type (33). LEMS is found in approximately 3% to 4% of patients with SCLC (33, 34), and is usually associated with the presence of voltage-gated calcium channel antibodies. When LEMS is detected in association with SCLC in its paraneoplastic state, autoantibodies to Hu-D and/or SOX2 are also found in most patients (22). We have been able to show in this study that exclusion of the LEMS patients' data from the overall analysis, which may have been expected to overestimate the presence of antibodies to such TAAs, did not significantly change the positivity of the assay for cancer detection.

A feature of immunosenescence is an increase in autoantibody levels to a variety of antigens with increasing age, as reported in several studies (35–37); in addition, women display a different pattern of autoimmune diseases relative to men (38). To achieve a reliable comparison between a cancer and a control population, patients with SCLC were therefore matched by age and gender to a normal healthy control population. Tobacco smoking has also been reported to alter an autoantibody profile (39); in fact, autoantibodies to p53 have been shown in individuals with chronic obstructive pulmonary disease (COPD) who smoke (20). In view of these findings, the smoking status of the control group was also closely matched to that of the test group to ensure that the results would be appropriate for a population of smokers or ex-smokers at a higher risk of developing lung cancer. We have also analyzed the presence of autoantibodies to these antigens in 103 individuals with LEMS who are at a particularly high risk for SCLC and did not find a statistically increased level of autoantibodies in this group over the normal matched population (data submitted).

A recent study which analyzed the presence of autoantibodies to 6 of the antigens investigated here reported the technical (16) and clinical (40) validation of an autoantibody test for all lung cancers, using a calibrated system. These publications reported that such a test could reproducibly detect nearly 40% of primary lung cancers (SCLC and NSCLC), with a specificity of 90% for both age-matched normal sera, and 89% for individuals with benign lung disease (40). With 5 of the same antigens, but with the inclusion of Hu-D, 55% of a much larger number of SCLCs could be detected at the same specificity of 90% and 42% of SCLCs at a higher specificity of 99%.

All 4 of the individuals who were considered to be at an increased risk of developing lung cancer due to the presence of a PND and their smoking history had raised levels of autoantibodies and are being closely monitored. In fact, one of the individuals within the stage IA cancer group was asymptomatic at time of blood collection and originally considered at risk of developing lung cancer due to this individual's sensory neuronopathy and smoking history. Following autoantibody results and subsequent CT, the individual was found to have 2 small lung nodules highly suspicious of SCLC. This has subsequently been clinically confirmed.

Previous publications (12–17, 19, 40) have highlighted the potential value of a panel of autoantibodies for the early detection and monitoring of cancer. Autoantibodies to individual antigens have also been described prior to clinical diagnosis of cancer (13, 18, 20, 41), with autoantibodies to p53 having been reported prior to diagnosis of lung cancer in smokers with COPD (20) or in patients with asbestosis (18). In the latter publication, 13 patients were shown to have p53 autoantibodies prior to clinical diagnosis of cancer with the average lead time (time from first positive sample to diagnosis) of 3.5 years (range = 1–12 years). Using a panel of antigens, autoantibodies have also been reported up to 5 years before screening by CT in lung cancer (13). This study has shown that elevated levels of autoantibodies are present at a similar frequency in individuals with all stages of disease ranging from stage IA tumors to those with ED. All 4 of the individuals who had samples taken between 1 and 3 months before first diagnosis of their SCLC also had elevated levels of autoantibodies, at a time when they did not have respiratory or systemic symptoms suggestive of cancer. These findings further support previous publications that show tumor-associated antibodies prior to clinical presentation of lung cancer.

Autoantibodies to the panel(s) of antigens reported in this study can be used to aid early identification of patients with SCLC in a high-risk population. Because of the large numbers of prospectively collected SCLC samples, appropriately matched controls, and the use of a validated, calibrated assay, we believe that the data reported in this article are the most statistically robust and clinically relevant data to date.

The sensitivity of 55% with a specificity of 90% is higher than that reported for mammography in high-risk young women (42). Furthermore, the sensitivity and specificity have to be seen in the context of a disease (i.e., SCLC) which is usually diagnosed late and has a mortality rate of greater than 90% worldwide but which may have a possibility for increased survival if diagnosed at an early stage while still localized. In contrast, annual screening by CT in the Mayo helical CT screening trial had a specificity of 49% for all types of lung cancer (with a sensitivity of 67%) in the prevalence round. The specificity decreased to 36% after 2 years and 25% after 4 rounds of screening by CT (43). Although such comparisons serve to highlight the potential value of an autoantibody test for lung cancer that has a specificity of 90%, the authors envisage autoantibody technology and imaging as being complementary.

An increase in specificity for cancer detection to 99% may be worth a reduction in sensitivity from 55% to 42% for SCLC, the exact specificity being led by the clinical utility of such a test. Ultimately, it would be envisaged that a simple, reliable, and noninvasive blood test that can aid the early detection of SCLC in a high-risk population could be used most effectively to direct imaging modalities with low specificity, such as spiral CT, and thereby in combination presents a real opportunity to impact on patient outcomes.

Disclosure of Potential Conflicts of Interest

C.J. Chapman is a consultant to Oncimmune Ltd. J.F.R. Robertson is a shareholder and consultant to Oncimmune Ltd., a University of Nottingham spinout company. The authors received other commercial grant support from Oncimmune Ltd.

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