

## **Autoantibodies in Lung Cancer – possibilities for early detection and subsequent cure.**

\*Caroline J Chapman PhD, \*\*Andrea Murray PhD, \*\*Jane E McElveen \*\*\* Ugur Sahin MD, \*\*\*Ulrich Luxemburger, \*\*\*Özlem Türeci MD; \*\*\*Rainer Wiewrodt MD, \*\*Anthony C Barnes PhD; \*/\*\* John F Robertson MD.

\*Division of Breast Surgery, The University of Nottingham, Nottingham, NG5 1PB, UK  
\*\* Oncimmune Ltd, Nottingham City Hospital, Nottingham, NG5 1PB, UK  
\*\*\* Department of Medicine III, Johannes Gutenberg University, Mainz, Germany

Correspondence and reprint requests should be addressed to: Dr Caroline Chapman; Division of Breast Surgery, Clinical Sciences Building, Nottingham City Hospital, Nottingham, NG5 1PB, UK. email: [caroline.chapman@nottingham.ac.uk](mailto:caroline.chapman@nottingham.ac.uk) . Phone +44 115 8231825

Running header: Autoantibodies in lung cancer

Keywords: Autoantibodies; Lung Cancer; Diagnosis; Tumor markers; Tumor Antigens

### **Abstract**

**Background.** Individuals with lung cancer usually present at a late stage in the course of their disease when their chances of long term survival are low. At present there is little to offer for early diagnosis, even in individuals at high risk of developing the disease. Autoantibodies have been shown to be present in the circulation of individuals with various forms of solid tumour before cancer-associated antigens can be detected, and these molecules can be measured up to 5 years before symptomatic disease. This study aims to assess the potential of a panel of tumour-associated autoantibody profiles as an aid to other lung cancer screening modalities.

**Methods.** Plasma from normal controls (n=50), patients with non small cell lung cancer (NSCLC) (n=82) and patients with small cell lung cancer (SCLC) (n=22) were investigated for the presence of autoantibodies to p53; c-myc; HER2; NY-ESO-1; CAGE; MUC1 and GBU4-5 by enzyme-linked immunosorbent assay.

**Results.** Elevated levels of autoantibodies were seen to at least 1/7 antigens in 76% of all the lung cancer patient plasma tested, and 89% of node negative patients, with a specificity of 92%. There was no significant difference between the detection rates in the lung cancer subgroups, although more individuals with squamous cell carcinomas (92%) could be identified.

**Conclusion.** Measurement of an autoantibody response to one or more tumour associated antigens in an optimised panel assay, could provide a sensitive and specific blood test to aid the early detection of lung cancer.

## Introduction

Lung cancer is the largest cause of death from cancer worldwide, killing around 900,000 people every year (1). Tobacco smoking is estimated to cause around 90 per cent of all cases in men and 80% in women (2,3) however other recognised risk factors for lung cancer include passive smoking; occupational exposures, especially to asbestos; and radon exposure (1). The latency period for lung cancers attributable to smoking is at least 20 years (1) yet lung cancer is often detected following chest X-ray by which time it is usually advanced and patients cannot be cured by treatment. Presently there is no accepted early diagnostic test although screening trials using spiral computed tomography (CT) in 'at risk' individuals are ongoing (4).

Since the 1970's there has been little improvement in the five-year survival rates for lung cancer, which are at their highest in the USA but are still only 13% for men and 17% for women (5). In contrast if lung cancer is diagnosed early the 5 year survival rate for UICC stages I and II is reported to be up to 50%, and for stage 0 (when the cancer is found only in a local area and only in the first few layers of cells) approaches 80%. There is therefore an urgent priority to produce a screening test which can identify patients with lung cancer in its early curable stage(s) (5).

There is an increasing body of literature describing the presence of a humoral immune response, in the form of autoantibodies, to tumour associated antigens (TAAs) in lung and other solid tumours (6-8) and these autoantibodies have been described as being present in individuals prior to symptomatic disease (6,9-11). We have recently hypothesized that the measurement of such tumour associated antibodies could be useful in breast cancer detection as a test to aid mammography, and have reported elevated levels of autoantibodies to any one of a selected panel of TAAs in the circulation of individuals with early invasive breast cancer, and ductal carcinoma in situ (DCIS), taken at disease diagnosis (12). This report expands on these findings in lung cancer and describes a highly sensitive Enzyme Linked Immunosorbent Assay (ELISA) which measures the presence of elevated levels of autoantibodies to TAAs in the peripheral blood of patients with both small cell (SCLC) and non-small cell lung cancer (NSCLC).

The panel of seven antigens selected in this study comprises a number of well-recognised cancer-associated proteins including the c-myc oncogene whose expression is observed in a wide variety of tumours, and the p53 tumour suppressor gene, that is mutated in a large number of cancers. p53 was the first antigen described to elicit autoantibodies in cancer (13) and autoantibodies to p53 have also been shown to be raised in individuals who smoke both with and without evidence of cancer (14,15). Such anti-p53 antibodies can, in some cases, be detected prior to the cancer diagnosis (11,16). The panel also includes antigens which are over-expressed (HER2) or aberrantly expressed (MUC1) on the cell surface of many solid tumours including lung, breast, stomach, prostate and ovary (17-19) and which have been shown to induce autoantibody responses in both breast (9,12) and in the case of MUC1 NSCLC (20). Also included are two previously described cancer testis antigens, NY-ESO-1 which has previously been shown to induce autoantibodies in NSCLC (21,22), and CAGE which has been described as capable of inducing an autoantibody response in gastric (23,24) pancreatic (25) and some lung cancers (21,23). The final antigen in the panel was a recently identified protein GBU4-5 which was identified using SEREX technologies (26) and described in a previous publication (24). GBU4-5 encodes a DEAD box domain (like CAGE), but until 2003 had not been described as eliciting an autoantibody response. Autoantibody responses to different antigens that encode DEAD box proteins have recently been described (22,25). These DEAD box-containing proteins are involved in RNA processing, ribosome assembly, spermatogenesis, embryogenesis, and cell growth and division (27) and these proteins may well be important in the carcinogenic pathway. They seem to be immunogenic, cancer specific and could provide diagnostic and potentially immunotherapeutic cancer targets.

## **Methods**

### **Blood samples & patient details**

Blood samples were collected from 104 patients with lung cancer who were recruited after histopathological confirmation of the tumour. The study was approved by the institutional ethics committee of Rheinland-Pfalz. The smoking status of these individuals was unknown. Samples (both pre and post treatment) were taken at various time points after diagnosis (0-36 months). Normal blood plasmas were also obtained from 50 healthy blood donors with no further clinical information; the age, sex and smoking status of these individuals was unknown.

### **Antigen Production**

Specific cDNAs for p53, HER2 (extracellular domain), c-myc, NY-ESO-1, CAGE and GBU4-5 were subcloned, along with a small tag, into the Pet21b expression vector (Novagen, Darmstadt, Germany). The recombinant proteins were expressed in BL21(DE3) bacteria (Novagen), grown in CYM media (28) and purified using His trap affinity columns (Amersham, Uppsala, Sweden) according to manufacturer's protocols. A negative control protein was also produced (small tag alone) and purified under identical conditions. Proteins were refolded according to the manufacturer's protocol and tested for purity by SDS-PAGE (silver and coomassie stained (28)) as well as by Western Blotting (14) with appropriate mouse monoclonal antibodies. Only proteins that were >95% pure were used in the assays. In this study assessment of MUC1 autoantibodies was made using a MUC1 'VNTR' (29) peptide (Peptide Protein Research Ltd, Fareham, UK) conjugated to BSA, with BSA alone acting as a negative control.

### **Autoantibody detection**

Autoantibody detection was by ELISA using microtitre plates coated with recombinant antigen or peptide according to in-house protocols (patent pending). Remaining binding sites were blocked with High Salt Buffer (HSB: Phosphate Buffered Saline + 0.5M NaCl, 0.2% w/v casein, 0.05% Tween 20). For all assays, freshly thawed plasma samples (diluted 1/100 in HSB) were incubated in triplicate at 50µl per well for 90 min, as well as appropriate control mouse monoclonal antibodies specific for capture proteins. Horseradish peroxidase (HRP) conjugated rabbit anti-human IgG/M/A/kappa (Stratech, Soham, UK) or anti-mouse Ig antibodies (Dako, Ely, UK) were used as secondary antibodies at the dilution recommended by the manufacturer. Ready prepared 3,3',5,5'-tetramethylbenzidine (TMB, Chemicon, Chandlers Ford, UK) was used as the chromogenic substrate for HRP and absorbance values were determined after a 10 minute period at A<sub>650nm</sub>. All incubations were carried out with shaking at room temperature and plates were washed 3, times with PBS containing Tween 20 (0.1% v/v, Sigma, Poole, UK) between each step.

### **Statistics**

Standard descriptive statistics such as frequencies, mean, and SD were calculated to describe the study population. All analyses were performed using Microsoft Excel or Graph Pad software and were regarded as explorative. The number and proportion of positive samples were presented with 95% exact confidence interval (95% CI) for binomial proportions (30). Chi-squared tests were used to flag where the proportion of positive results was significantly different between cancer groups and the normal controls. For the panel 1 of 7, testing was also performed to look at differences in positivity between lung cancer subgroups. This analysis was repeated on two subsets of data which consisted of pre-treatment samples taken up to one and six months of diagnosis.

### **Autoantibody Assays**

Positive seroreactivity was defined as an optical density (OD) value greater than either the mean plus 2 or mean plus 3 standard deviations (SD) of the normal cohort. The more stringent cut-off (3SD) was applied to the c-myc, NY-ESO-1, p53, HER2 and MUC1 autoantibody assays,

incorporating on average 99% of the distribution of the data. A cut-off value of an OD value greater than the mean +2SD of the normal population was applied to the autoantibody assays using the SEREX-identified antigen GBU4-5 and CAGE (24). In all autoantibody assays on normal plasma, values that were greater than the mean +5SDs were removed to produce cut-off values, but were included in analysis of specificity.

All assays were performed in triplicate on between two to four separate occasions. In addition all positive plasma were confirmed with repeat testing, as were some negative plasma. Samples were designated positive for each separate autoantibody assay if there was a reproducible signal above the cut-off level of the normal group. For example in lung cancer patients a sample was only deemed reproducibly positive for an autoantibody assay if at least 2 out of 2 or 3 out of 4 inter-assay runs showed an elevated value above the cut-off. Where it was deemed that the result from one of the first runs was technically unassessable and where only 2 out of the remaining three assessable runs were elevated a fifth run was performed.

**Results**

Patient details and tumour characteristics are shown in Table 1 for both the total sample set (n=104), as well as those samples taken at or close to diagnosis (n=70). No further details on the normal samples were available.

**Table 1: Patient details and clinical characteristics of lung cancer samples.**

Table 1a. All samples. Table 1b\* Samples taken within 4 (SCLC) or 6 (NSCLC) months of diagnosis date. NOS not otherwise specified. LN Lymph Node

**Table 1a**

All Samples (n=104)	NSCLC	SCLC
Total Number (%)	82 (100%)	22 (100%)
Squamous cell carcinoma	25 (31%)	n/a
Adenocarcinoma	35 (43%)	n/a
Mixed (Squ & Adeno)	1 (1%)	n/a
Large Cell carcinoma	1 (1%)	n/a
NOS	20 (24%)	n/a
Mean Age +/-SD (years)	64 +/- 10	62 +/-11
Age range (years)	36-83	44-84
% Male	70%	64%
% LN Negative	15%	0

**Table 1b**

Defined Samples* (n=70)	NSCLC	SCLC
Total Number (%)	55 (100%)	15 (100%)
Squamous cell carcinoma	16 (29%)	n/a
Adenocarcinoma	24 (44%)	n/a
NOS	15 (27%)	n/a
Stage I	5 (9%)	0%
Stage II	4 (7%)	0%
Stage III	13 (24%)	3 (20%)
Stage IV	27 (49%)	12 (80%)
NOS	4 (7%)	1 (7%)
Mean Age +/-SD (years)	63 +/- 11	61 +/-10
Age range (years)	36-83	52-75
Male	38 (70%)	10 (67%)
Pretreatment samples	34 (62%)	13 (87%)

Table 2 shows levels of detection of autoantibodies against individual antigens in the two lung cancer disease groups and normal blood donor controls. The percentage of positive results in individual autoantibody assays ranged between 5% (NY-ESO-1) and 36% (MUC1) in the SCLC group and between 10% (p53) and 34% (MUC1) in the NSCLC group compared to between 0% (GBU 4-5, c-myc and MUC1) and 4% (CAGE) in the normal controls. The highest level of autoantibody sensitivity was seen to the MUC1 peptide antigen for both forms of lung cancer. Specificity of the assay was calculated as the percentage of true negatives that correctly gave a negative result. Individual assay specificity for each antigen varied from 96-100%.

**Table 2: Frequency of autoantibodies to tumour-associated antigens**

Group	Total	Number and percentage positive (with 95 % Exact Confidence Interval)								Panel 1/4	Panel 1/7	
		CAGE	GBU 4-5	HER2	p53	c-myc	NY-ESO-1	MUC1				
All LC	n 104	29***	21***	13**	12*	12***	19***	36***			71***	79***
	%+ve (95% CI)	28 (20, 38)	20 (13, 29)	13 (7, 20)	12 (6, 19)	12 (6, 19)	18 (11, 27)	35 (26, 45)			68 (58, 77)	76 (67, 84)
SCLC	n 22	5*	4**	4*	4*	2*	1 NS	8***			12***	15***
	%+ve (95% CI)	23 (8, 45)	18 (5, 40)	18 (5, 40)	18 (5, 40)	9 (1, 29)	5 (0, 23)	36 (17, 59)			55 (32, 76)	68 (45, 86)
NSCLC	n 82	24***	17***	9*	8 NS	10***	18***	28***			59***	64***
	%+ve (95% CI)	29 (20, 40)	21 (13, 31)	11 (5, 20)	10 (4, 18)	12 (6, 21)	22 (14, 32)	34 (24, 45)			72 (61, 81)	78 (68, 86)
Normal	n 50	2	0	1	1	0	1	0			3	4
	%+ve (95% CI)	4 (0, 14)	0 (0, 7)	2 (0, 11)	2 (0, 11)	0 (0, 7)	2 (0, 11)	0 (0, 7)			6 (1, 17)	8 (2, 19)
Specificity	% 50	96	100	98	98	100	98	100			94	92

Number (n) and percentage positivity (% +ve) with 95 % confidence interval (95% CI) in each patient group. Positivity was defined as a value greater than the mean of the normal population plus 2SD for GBU4-5 and CAGE, or mean plus 3SD for the other assays. All LC: all lung cancer samples tested. SCLC: small cell lung cancer. NSCLC: non small cell lung cancer. Normal: Normal blood donor plasma. Panel 1/4 or 1/7 denotes raised levels of at least 1 autoantibody of the panel. Panel of 4: CAGE, GBU4-5; NY-ESO-1, MUC1 antigens only. \* denotes p-value relative to normal control plasma. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , NS - not significant  $p > 0.05$  (Chi-squared analysis)

The lung cancer patients (SCLC and NSCLC subgroups) were significantly different from the normal controls for all autoantibody assays ( $p < 0.05$ ) apart from p53 in NSCLC and NY-ESO-1 in the case of SCLC. Only 4 of the normal cohort (8%) had elevated levels of autoantibodies to any one of the tumour associated antigens (TAAs) and one of these also had autoantibodies to multiple tumour associated antigens (p53, c-myc, HER2 and CAGE) (Figure 1) which is rarely observed in normal samples (12). No clinical data was available on these 4 individuals.

Combination of these 7 autoantibody assays led to an enhancement in autoantibody detection of cancer with a panel sensitivity of 76% and panel specificity of 92%. In the majority of cases the levels of autoantibody responses to individual antigens in the cancer cases were significantly different to the normal control plasma ( $p < 0.05$ ) but their importance in the panel assay varied. For example raised levels of autoantibodies to the HER2 antigen were observed in 12% of the lung cancers; however if the HER2 antigen was removed from the panel the detection rate and overall specificity for the NSCLC samples remained the same, and the overall sensitivity of the panel for all lung cancers only dropped from 76% to 74%. This was reflected by the observation that a restricted panel assay consisting of the four antigens CAGE, GBU4-5, NY-ESO-1 and MUC1 exhibited only a slightly reduced sensitivity of 68% with an increased specificity of 94% (Table 2).

In almost half of the seropositive individuals in Panel 1 of 7, autoantibodies were raised to a second antigen with similar patterns in both NSCLC and SCLC samples, whilst only 1 of the 50 normal plasma samples exhibited more than 1 autoantibody response (Figure 1).

**Table 3: Autoantibody panel sensitivity by lung cancer subgroup**

Group		Autoantibody positivity population (panel of 7)		
		All samples 0-36 months	Subset 0-1 0-1 month PreTx	Subset 0-6 0-6 months PreTx
SCLC	number +ve	15/22	5/8	9/13
	% +ve (95% CI)	68 (45, 86)	63 (24, 91)	69 (39,91)
NSCLC Squamous	number +ve	23/25	5/5	11/12
	% +ve (95% CI)	92 (74, 99)	100 (48, 100)	92 (62,100)
NSCLC Adeno	number +ve	27/35	5/7	13/16
	% +ve (95% CI)	77 (60, 90)	71 (29, 96)	81 (54,96)
NSCLC NOS	number +ve	14/22	0/2	2/6
	% +ve (95% CI)	64 (41, 83)	0 (0, 84)	33 (4,78)
All LC	number +ve	79/104	15/22	35/47
	% +ve (95% CI)	76 (67,84)	68 (45, 86)	74 (60, 86)
<b>p-value from chi-squared test #</b>		0.1065	0.2108	0.2848

Number and percentage positivity (% +ve) with 95 % confidence interval (95% CI) in each patient group using Panel1/7. Analysis repeated on two subset sample populations taking only pre-treatment (pre Tx) samples up to 1 month and 6 months from diagnosis.

All LC: all lung cancer samples tested. SCLC: small cell lung cancer. NSCLC: non small cell lung cancer. Adeno: adenocarcinoma. Squamous: squamous cell carcinoma. NOS: not otherwise specified. # Chi squared test across cancer sub groups noting that NOS was pooled with Adeno in the subset analyses due to low numbers.

The sensitivity of 76% (95% CI: 67%, 84%) that was achieved by Panel 1/7 in all 104 LC samples was similar for the subsets of data where samples were taken pre-treatment, with sensitivity estimated at 68% (95% CI: 45%, 86%) and 74% (95% CI: 60%, 86%) for samples within one and six months of diagnosis, respectively (Table 3). For all samples, sensitivity ranged from 64% (95% CI: 41%, 83%) for the NSCLC NOS group to 92% (95% CI: 74%, 99%) for the NSCLC squamous group. Similar patterns were seen in the subsets. However, the differences in sensitivity between cancer sub groups was only borderline significant (p=0.1065 for all samples). There was also no apparent difference in detection rates if the lung cancer samples were subdivided by patient age or sex (data available in web appendix).

95% confidence intervals revealed that even when sample numbers were small, eg in pretreatment squamous cell carcinoma samples taken within 6 month of diagnosis (n=12), it would be expected that over 62% of cases would have elevated levels of autoantibodies using this panel of 7 antigens (95%CI: 62%, 100%). When all the squamous cell carcinoma samples were analyzed this 92% detection rate was maintained but with increased confidence in the result (23/25 autoantibody positive samples, 95% CI: 74%, 99%) (Table 3).

The levels of sensitivity appeared to be maintained regardless of the lung cancer stage though further confirmatory data is required (table 4). All 3 stage I and all 3 stage II pretreatment NSCLCs were detectable giving a sensitivity estimated at 100%, but the wide confidence interval (95% CI: 29%, 100%) reflects the limited samples and need for further data. Eight out of nine lymph node negative pre treatment NSCLC samples also had elevated levels of autoantibodies leading to an estimated sensitivity of 89% (95% CI: 52%, 100%) (data not tabulated).

**Table 4: Autoantibody assay panel sensitivity by tumour stage.**

Group	subgroup		Autoantibody positivity population (panel of 7)			
			Stage I	Stage II	Stage III	Stage IV
SCLC	n=14	number +ve	0	0	2/3	7/11
		%+ve (95% CI)			67 (9, 99)	64 (31, 89)
SCLC	PreTx n=12	number +ve	0	0	1/1	7/11
		%+ve (95% CI)			100 (3, 100)	64 (31, 89)
NSCLC	n=49	number +ve	4/5	4/4	10/13	21/27
		%+ve (95% CI)	80 (28, 99)	100 (40, 100)	77 (46, 95)	78 (58, 91)
NSCLC	PreTx n=33	number +ve	3/3	3/3	5/7	15/20
		%+ve (95% CI)	100 (29, 100)	100 (29, 100)	71 (29, 96)	75 (51, 91)

Number and percentage positivity (% +ve) with 95 % confidence interval (95% CI) in each tumour stage (where known) using Panel1/7. Stage taken at diagnosis date follows from T,N,M-Staging. SCLC: small cell lung cancer. NSCLC: non small cell lung cancer. 95% CI denotes the 95% confidence interval. PreTx – pretreatment samples. Samples taken within 6 months of diagnosis for NSCLC, and within 4 months of diagnosis for SCLC.

### Discussion

Autoantibodies were produced to all seven of the TAAs studied. The presence of raised levels of autoantibodies to the known tumour associated antigens p53, c-myc, HER2, MUC1 and NY-ESO-1 were in broad agreement with published data for individual autoantibody assays in both breast (reviewed in 8) and other cancers such as colorectal, gastric, prostate, liver, and lung (7,31,32) and confirms the finding of ourselves and others that although measurement of autoantibodies to a single tumour associated antigen is possible, the low sensitivity renders single autoantibody measurements of little use for screening and early diagnosis of cancer. Combination of these autoantibodies, together with the SEREX-identifiable antigens CAGE and GBU4-5 into a panel assay test provides an excellent level of sensitivity for the detection of lung cancer. Analysis of autoantibodies to the full panel of 7 antigens resulted in ¾ of all lung cancer samples being identified, and 92% of squamous cell carcinomas with a specificity for cancer of 92% making this a test with potential clinical impact.

A previous study by Zhang et al (7) demonstrated autoantibody reactivity to a panel of seven cancer associated antigens – which differed from the antigens investigated here in all but p53 and c-myc. Their reported levels of sensitivities and specificities in lung cancer were 68% and 89-90% (depending on the normal group) respectively, which are similar to those we have demonstrated. However the study numbers were smaller than that those used here and there was no data on the stage, grade or subtype of the cancers tested.

This level of sensitivity of the assay for the identification of lung cancer was maintained in the subset of samples taken at or within 1 month of their cancer diagnosis. As it is unlikely that these autoantibodies were generated at the time of clinical presentation, the implications are that the antibodies would be present prior to diagnosis. There is published evidence that autoantibodies can be detected a number of years before clinical symptoms are observed (9,11,16) and affirmation of the results presented here, in prediagnostic samples, would confirm the utility of such an autoantibody test in lung cancer.

Although almost all the autoantibody assays measured significant responses in the plasma of cancer patients, the individual sensitivity of each assay within the panel varied. Measurement of p53, c-myc and HER2 did not add significantly to the panel assay and increased sensitivity may be possible via substitution of these more general cancer antigens by other lung cancer specific

antigens in a panel assay. In contrast measurement of the autoantibody response to the MUC1 core peptide was integral to the panel assays and was the most sensitive assay in all the histological subtypes of the lung samples studied. Measurement of the autoantibody responses to the cancer testis antigens was also important. A previous study investigated the presence of autoantibodies to NY-ESO-1 antigen in NSCLCs and SCLCs and observed similar levels of sensitivity and specificity as reported here, finding SCLC patients more rarely having antibodies to NY-ESO-1 than NSCLC (19). In NSCLC the expression of NY-ESO-1 has been reported to be an independent prognostic marker of worse outcome in cases with adenocarcinoma (33), clinical follow-up data was not available on the population of patients reported in this study to confirm this previous finding.

Autoantibody responses to the DEAD-box proteins CAGE and the novel cDNA GBU4-5 were also highly sensitive and specific in this study. The DEAD-box cancer testis antigen CAGE (DDX58) has previously been shown to be expressed in a number of cancers including gastric, cervical and lung cancer tissue and cell lines, and autoantibodies have been reported to this protein in some but not all of the cancers samples studied (21), whereas autoantibodies to GBU4-5 have not been investigated in other cancers. We have preliminary data on the presence of autoantibodies to CAGE and GBU4-5 in individuals with primary invasive breast cancer (n=51, full manuscript in preparation). These data did not show elevated levels of autoantibodies to these antigens when compared to age matched normal sera (n=31) (CAGE: sensitivity 6%, specificity 97%; GBU4-5: sensitivity 4%, specificity 94%). These observations suggest that distinguishing different solid tumour types may be aided by measuring autoantibodies to different DEAD-box encoding proteins within a panel assay, although more data would be needed to confirm this.

Other antigens such as p53 and c-myc are more general cancer antigens and autoantibody responses to p53 have been reported to be raised in some individuals who smoke (14). At this time it is difficult to be certain whether p53 autoantibodies reflect very early cancers or alternatively may merely point to a cancer risk rather than the presence of disease. The smoking status of our normal and cancer subjects was unknown, however the p53 responses in this study were low and removal of this antigen from the panel was not found to significantly change the sensitivity or specificity of the panel for lung cancer. Autoantibodies to cancer associated antigens other than p53, in individuals who smoke have not been reported.

Elevated levels of autoantibodies, and particularly multiply elevated autoantibodies were rarely seen in these normal plasma, and this low level of tumour-associated autoantibodies is in agreement with other publications (7,12,22). No further information was available for the control group, including smoking history, so the possibility that one or more of these individuals could have been harboring an occult malignancy cannot be ruled out.

The potential clinical significance of autoantibodies for screening and early diagnosis appears to lie in combining an optimised panel of assays to measure these autoantibodies. The advantage of this approach is that it should be possible to detect the full range of heterogeneous lung cancers by increasing the number of autoantibody assays or altering the antigens used in the autoantibody panel, and with the evidence for autoantibodies present to antigens such as livin, survivin and annexins I & II, p62 and Hsp40 (7,34-37) as well as an increasing number of other cancer-specific immunoreactive antigens being described every year, a potential diagnostic assay using measurement of autoantibodies in at risk individuals should be available within a short period of time.

A diagnostic test for lung cancer is of particular importance due to the late stage at which patients currently present with this disease and the fact that this disease will cause significant

social burden for at least 20 years, even if all smoking was discontinued today. Obviously the potential harmful effects of any test must be taken into account when deciding on its suitability. The lungs are very sensitive to radiation so frequent X-rays are not be an ideal screening test. For screening to be introduced, a test needs to be simple, quick, inexpensive and beneficial so that it can be used on individuals at high risk of cancer development. A blood test, such as that described here is non-invasive, cost-effective relative to imaging tests, carries no side effects and is acceptable to the vast majority of patients.

The levels of sensitivity and specificity demonstrated here suggest that the measurement of autoantibodies could act as an aid to cancer diagnosis. Such a test could be performed on the peripheral blood of individuals with an increased risk of developing cancer as a means of selecting patients for an imaging test, such as spiral CT/ MRI. Persons with a positive autoantibody test would proceed to spiral CT/MRI, which if it confirmed a lung (or other) cancer would then lead to treatment. Imaging negative patients would continue to be intensively followed up in specialist clinics.

Further studies are planned using an increased assay panel which will include some tumour-associated antigens though to be more lung cancer specific. These will be tested on samples taken from earlier stage cancers as well as pre-diagnostic samples using age matched controls, with known smoking histories.

#### **Acknowledgements**

The authors would like to thank Natalie Colborne for technical assistance, and Susan McKendrick, CStat, for statistical advice. The authors are extremely grateful to European Union 5<sup>th</sup> Framework (EUCIP), Oncimmune Ltd and The University of Nottingham for financial support.

#### **Conflict of Interest**

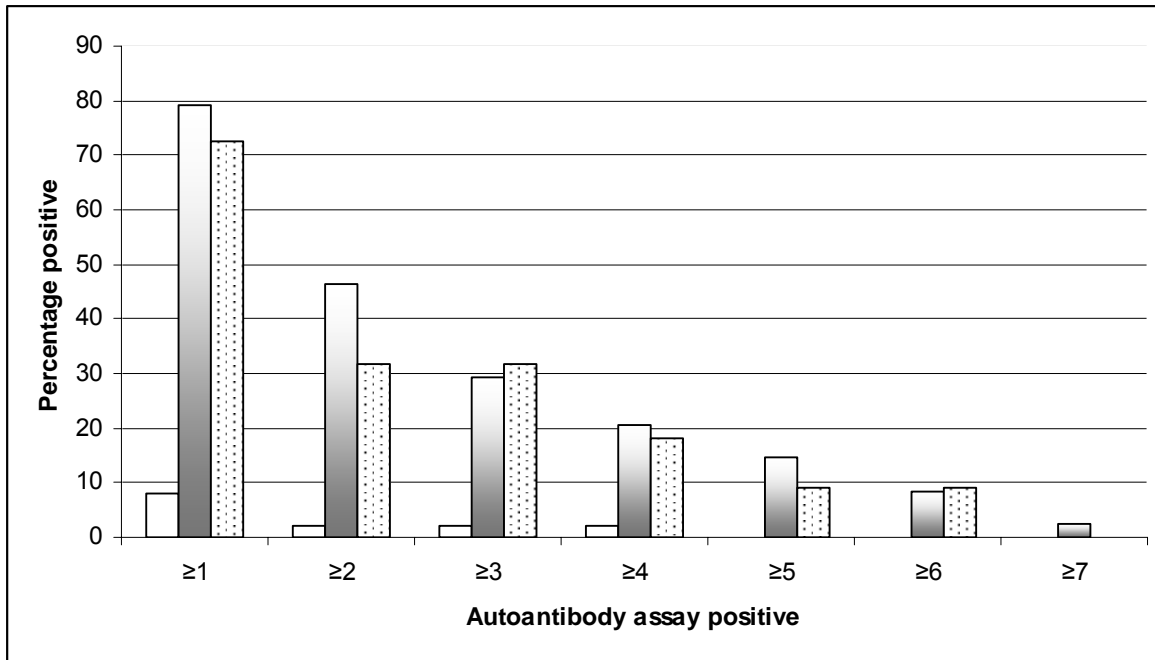
Oncimmune is a spinout company from the University of Nottingham. Professor John Robertson, the founder of the technology, is a shareholder and director of the company. Dr Caroline Chapman is a lecturer at the University of Nottingham and consults for Oncimmune. Dr Andrea Murray is an employee of Oncimmune while Dr Barnes is a shareholder. Jane McElveen is an employee of Oncimmune.

The Corresponding Author has the right on behalf of all authors and does not grant on behalf of all authors, an exclusive licence (or non-exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its Licensees to permit this article to be published in Thorax editions and any other BMJPGJL products to exploit all subsidiary rights, as set out in our licence <http://thorax.bmjournals.com/iflora/licence.pdf>

**Figure 1: Autoantibody panel assay sensitivity.**

Percentage of each patient group positive for 1 or multiple autoantibodies. Positivity was defined as a value greater than the mean of the normal population plus 2SD for the SEREX identified antigens CAGE & GBU4-5 or mean plus 3SD for the other assays. . SCLC: small cell lung cancer. NSCLC: non small cell lung cancer.

Key:



## References

1. Office for National Statistics. Twentieth Century Mortality - 95 years of mortality data in England and Wales by age, sex, year and underlying cause. ISBN 1 857742 397, 1997
2. Felip E, Pavlidis N & Stahel RA (coordinating authors for the ESMO guidelines task force. ESMO Minimal Clinical Recommendations for diagnosis, treatment and follow-up of small-cell lung cancer (SCLC) *Annals Oncol*, 16:i30-i31, 2005 (suppl 1)
3. Felip E, Stahel RA & Pavlidis N (coordinating authors for the ESMO guidelines task force. ESMO Minimal Clinical Recommendations for diagnosis, treatment and follow-up of non-small-cell lung cancer (NSCLC) *Annals Oncol*,16:i28-i29, 2005 (suppl 1)
4. MacRedmond R, McVey G, Lee M, et al. Screening for lung cancer using low dose CT scanning: results of 2 year follow up. *Thorax*. 61:54-56, 2006
5. Ries LAG, Eisner MP, Kosary CL, et al. (eds). SEER Cancer Statistics Review, 1975–2001. Bethesda, MD: National Cancer Institute.
6. Zhong L, Coe SP, Stromberg AJ, et al. Profiling tumor-associated antibodies for early detection of non-small cell lung cancer. *J Thor Oncol* 1:513-519, 2006
7. Zhang JY, Casiano CA, Peng XX, et al. Enhancement of antibody detection in cancer using panel of recombinant tumor-associated antigens. *Cancer Epidemiol Biomarkers Prev* 12: 136-143, 2003
8. Storr SJ, Chakrabarti J, Barnes A, et al. Use of autoantibodies in breast cancer screening and diagnosis. *Expert Rev Anticancer Ther*; 6:1215-1223, 2006
9. Robertson JFR, Chapman C, Cheung K-L, et al. Autoantibodies in early breast cancer. *J Clin Oncol*; 23: 549. 2005 Suppl 1
10. Robertson JFR, Graves RL, Price MR, Inventors; OncoImmune Ltd, assignee. Tumour markers. European patent EP-B 1078264, 2005
11. Li Y, Karjalainen A, Koskinen H, et al. p53 autoantibodies predict subsequent development of cancer. *Int J Cancer* 114:157-160, 2005
12. Chapman C, Murray A, Chakrabarti J, et al. Autoantibodies in Breast Cancer: their use as an aid to early diagnosis. *Annals of Oncology* 18: 868-73, 2007
13. Crawford LV, Pim DC, Bulbrook RD. The detection of antibodies against the cellular protein p53 in sera from patients with breast-cancer. *Int J Cancer* 30:403-408, 1982
14. Sangrajrang S, Sornprom A, Chernrungrroj G, et al. Serum p53 antibodies in patients with lung cancer: correlation with clinicopathologic features and smoking. *Lung Cancer* 39:297-301, 2003
15. Li Y, Brandt-Rauf PW, Carney WP, et al. Circulating anti- p53 antibodies in lung cancer and relationship to histology and smoking. *Biomarkers* 4:381-390, 1999
16. Trivers GE, DeBenedetti VM, Cawley HL, et al. Anti p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer. *Clin Cancer Res* 2:1767-1775, 1996

17. Veale D, Ashcroft T, Marsh C, et al. Epidermal growth factor receptors in non-small cell lung cancer. *Br J Cancer*. 55:513-516, 1987
18. Szelachowska J, Jelen M, Kornafel J. Prognostic significance of intracellular laminin and Her2/neu overexpression in non-small cell lung cancer. *Anticancer Res* 26: 3871-3876. 2006
19. Awaya H, Takeshima Y, Yamasaki M, et al. Expression of MUC1, MUC2, MUC5AC, and MUC6 in atypical adenomatous hyperplasia, bronchioloalveolar carcinoma, adenocarcinoma with mixed subtypes, and mucinous bronchioloalveolar carcinoma of the lung. *Am J Clin Pathol* 121:644-653, 2004
20. Hirasawa Y, Kohno N, Yokoyama A, et al. Natural autoantibody to MUC1 is a prognostic indicator for non-small cell lung cancer. *Am J Respir Crit Care Med* 161:589-1594, 2000
21. Krause P, Tureci O, Micke P, et al. SeroGRID: an improved method for the rapid selection of antigens with disease related immunogenicity. *J Immunol Methods* 283:261-7, 2003
22. Türeci O, Mack U, Luxemberger U, et al. Humoral responses of lung cancer patients against tumor antigen NY-ESO-1. *Cancer Lett* 236:64-71 2006
23. Cho B, Lim Y, Lee DY, et al. Identification and characterization of a novel cancer/testis antigen gene CAGE. *Biochem Biophys Res Commun* 292:715-26, 2002
24. Cho B, Lee H, Jeong S, Bang YJ, Lee HJ, Hwang KS, Kim HY, Lee YS, Kang GH, Jeung DI. Promotor hypomethylation of a novel cancer/testis antigen gene CAGE is corelated with its abberant expression and is seen in premalignant stage of gastric cancer. *Biochem Biophys Res Commun* 307:52-63, 2003
25. Xia Q, Kong XT, Zhang GA, et al. Proteomics-based identification of DEAD-box protein 48 as a novel autoantigen, a prospective serum marker for pancreatic cancer. *Biochem Biophys Res Commun* 330:526-532, 2005.
26. Tureci O, Usener D, Schneider S, Sahin U. Identification of tumor-associated autoantigens with SEREX. *Methods Mol Med*. 109:137-54. 2005
27. Linder P. Dead-box proteins: a family affair--active and passive players in RNP-remodeling. *Nucleic Acids Res* 34:4168-4180, 2006
28. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning a laboratory manual*. (ed 2) (USA): Cold Spring Harbour Laboratory Press; 1989.
29. Gendler SJ, Spicer AP, Lalani EN et al. Structure and biology of the carcinoma-associated mucin, MUC1. *Am Rev Resp Dis* 144: S42-47. 1991
30. Armitage P, Berry G, Matthews JNS. *Statistical Methods in Medical Research*. 4th Edition. Blackwell Science, Oxford, UK (Section 4.4, p117) 2002.
31. Nesterova M, Johnson N, Cheadle C, et al. Autoantibody biomarker opens a new gateway for cancer diagnosis. *Biochimica et Biophysica Acta* 1762:398–403, 2006
32. Neri M, Betta P, Marroni P, Filiberti R, Cafferata M, Mereu C, Ivaldi G, Montanaro F, Puntoni R, Paganuzzi M. Serum anti-p53 autoantibodies in pleural malignant mesothelioma, lung cancer and non-neoplastic lung diseases. *Lung Cancer* 39:165-72, 2003
33. Gure AO, Chua R, Williamson B, et al. Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clin Cancer Res*11:8055-8062, 2005

34. Migliorino R, Shi FD, Peng XX, et al. Autoimmune response to anti-apoptotic protein survivin and its association with antibodies to p53 and c-myc in cancer detection. *Cancer Detect Prev* 29:241-248, 2005
35. Brichory FM, Misek DE, Yim AM, et al. An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *PNAS* 98:9824-9829, 2001
36. Yagihashi A, Asanuma K, Kobayashi D, et al. Detection of autoantibodies to livin and survivin in sera from lung cancer patients. *Lung Cancer* 48 217-221 2005.
37. Oka M, Sato S, Soda H, et al. Autoantibody to heat shock protein Hsp40 in sera of lung cancer patients. *Jpn J Cancer Res* 92:316-320, 2001