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Lung cancer – can autoantibodies provide an aid to diagnosis?

Kelly M Woodard & Caroline J Chapman†
†*The University of Nottingham, Division of Breast Surgery, Clinical Sciences Building, Nottingham City Hospital, Nottingham NG5 1PB, UK*

Background: More publications are describing autoantibodies specific to tumour-associated antigens in a range of solid tumours, including lung cancer. Importantly, these antibodies have even been described as being detectable before the tumour becomes clinically evident, making them interesting targets for use as novel biomarkers in lung cancer. *Objective*: This review provides an overview of the current literature regarding autoantibodies in lung cancer, and addresses the key question of whether the measurement of circulating autoantibodies can be utilised in lung cancer for diagnosis, screening, prognosis and monitoring clinical outcome. *Methods*: The literature used in this review was retrieved from PubMed using a combination of the following keywords: antigen array, autoantibodies, diagnosis, low-dose spiral computed tomography, lung cancer, non-small cell lung cancer, panel assay, prognosis, protein microarray, risk factors, SEREX, screening, small cell lung cancer and tumour-associated antigen. *Results*: Autoantibodies that react with autologous cellular antigens have been described in pre-diagnostic and diagnostic samples from patients with small cell, non-small cell and other forms of lung cancer, and can sometimes be correlated with clinico-pathological parameters. *Conclusion*: The measurement of autoantibodies to a panel of tumour-associated antigens has the potential to provide clinicians with the opportunity to detect early amplification of the carcinogenic signal, thereby providing a sensitive, specific and simple screening tool for the early diagnosis and subsequent early clinical intervention of lung cancer.

Keywords: antigen panel, autoantibodies, diagnosis, lung cancer, prognosis screening, protein microarray, tumour-associated antigen

1. Introduction

Lung cancer is the most common and lethal malignancy in the world with 1.3 million new cases diagnosed annually and over 1 million deaths each year [1]. In Europe it accounts for 13% of all new cancer cases [2], and in the UK it is the second most common cancer after prostate cancer in males, and the third most common cancer in females after breast and bowel cancer [3-6]. Lung cancer affects three to four times more men than women at present [7]; however the incidence among females is rising and is more than likely a reflection of increases in female smoking habits.

Lung cancer is rarely diagnosed in people younger than 40, but incidence rises steeply thereafter, peaking at around 75 – 84 years, with most cases (85%) occurring in people over the age of 60 [3-6]. Lung cancer was once considered a cancer of the developed world, but incidence patterns have changed dramatically, and now almost half of new lung cancer cases occur in the developing world [1]. Lung cancer is histologically subcategorised into non-small cell lung carcinomas (NSCLC), which make up ~ 80% of all lung cancers [7], and small cell lung

Table 1. Lung cancer prognosis according to stage.

Stage		TNM classification	5-year survival rate (%)	Ref.
NSCLC	IA*	T1N0M0	67	[7]
	IB*	T2N0M0	57	[7]
	IIA*	T1N1M0	55	[7]
	IIB*	T2N1M0, T3N0M0	38 – 39	[7]
	IIIA	T3N1M0, T1-3N2M0	15 – 30 (resectable tumour) 10 – 20 (unresectable tumour)	[16]
	IIIB	T1-4N3M0, T4N1-2M0	3 – 7	[7]
	IV*	T1-4N1-3M1	1	[7]
SCLC	Limited disease‡		15 – 25	[16]
	Extensive disease		2	[8]

*Only 20 – 25% of patients present at stages I and II and 50% present at stage IV [7].
‡Approximately 30% of patients present with limited disease [8].
NSCLC: Non-small cell lung carcinoma; SCLC: Small cell lung carcinoma; TNM: Tumour-nodes-metastasis.

carcinomas (SCLC), which comprise about 13 – 15% [8]. SCLCs exhibit a much quicker growth than NSCLCs, and often show an early haematogenous spread contrasted with a later lymphatic spread in NSCLC [7]. Although there are continuing advances in the development of therapeutic agents available for treatment of cancers, the 5-year mortality rate for lung cancer remains high and is between 85 and 90% [9].

The predominant risk factor for lung cancer is smoking [10], and it is thought to be the cause of 90% of all cases in men and 80% in women [11,12], with rates of lung cancer occurrence often lagging smoking rates by 20 – 30 years [9]. It has previously been shown that the relative risk of smoking on lung cancer is dependent on sex and race, with women being more susceptible to the carcinogenic risks of tobacco than men [13,14]. Hormonal and genetic factors may explain these observed risk differences and highlight the complex gene-environment interactions associated with malignancy; however a recent article by Freedman and colleagues suggests otherwise [15]. They analyzed cigarette smoking and subsequent lung cancer risk in a cohort of 463,837 individuals followed up from 1995 – 1996 until the end of 2003; their data suggested that women and men are at a similar risk for the development of lung cancer due to smoking, especially in present heavy smokers.

Passive smoking is a known risk factor for lung cancer and ~ 3000 adults die each year from lung cancer through passive smoking alone [16]. Dose–response relationships between the duration and intensity of exposure to spousal and workplace environmental tobacco smoke have been investigated and a weak statistical correlation has been observed [17].

Other recognised risk factors for lung cancer include exposure to asbestos, radon, nickel, chromium, bischloromethylether, arsenic, vinyl chloride and ionising radiation [16,18]. Studies investigating the effect on relative risk of lung cancer in people exposed to more than one risk factor have been reported, and perhaps the most interesting finding so far is the synergistic effect on lung cancer risk resulting from occupational exposure to asbestos combined with a smoking history [19-22].

Lung cancer is routinely detected by chest radiography, however other imaging modalities are often used and include magnetic resonance imaging (MRI) and low-dose computed spiral tomography (LDCT). On detection of suspicious lung lesions, histological analysis is carried out on small biopsy samples and a definitive diagnosis made. Occasionally, cytological analysis of cells obtained from sputum samples is used to assist in the diagnosis of lung cancer [23].

The prognosis of lung cancer patients is dependent on the tumour type (NSCLC or SCLC) and the tumour stage at the time of diagnosis [7], and this is especially true in patients with NSCLC, as highlighted in **Table 1**. Lung cancer is a disease that is often diagnosed at a late stage when the patient has become symptomatic, and it is estimated that more than two-thirds of NSCLCs are associated with lymph-node metastases at the time of diagnosis [24], resulting in an average 5-year survival rate of 14% for most patients [25]. By contrast, if lung cancer is detected and diagnosed earlier, the 5-year survival rate increases significantly (**Table 1**). Like NSCLC, most patients diagnosed with SCLC present with advanced (extensive) disease, and only 30% present with early (limited) disease [8], making the prognosis for SCLC very poor, with 5-year survival rates of 15 – 25% and < 5% for patients diagnosed with limited and extensive disease, respectively [16].

The poor 5-year survival rates for lung cancer mean that there is an urgent need to develop a screening tool for early detection, particularly in high-risk patient groups (smokers and ex-smokers). The current focus of screening research is the use of low-dose spiral CT (LDCT), as several studies

125 have revealed that LDCT can detect lung cancer in asymptomatic patients with a much higher sensitivity than chest radiography [26,27], with the Early Lung Cancer Action Project (ELCAP) demonstrating a 23% (233 out of 1000) and 2.7% (27 out of 1000) detection rate of small non-
130 calcified pulmonary nodules and malignant disease, respectively, in asymptomatic patients compared with 7% (68 out of 1000) and 0.7% (7 out of 1000) with chest radiography [26,28]. However, results between studies are contrasting, and a British study carried out by MacRedmond
135 and colleagues detected malignant disease in only 0.46% (2 out of 449) of patients investigated [29]. In addition to the ELCAP studies, Henschke and colleagues in the International Early Action Lung Cancer Action Project (I-ELCAP) have assessed survival rates of patients with
140 stage I lung cancer detected using LDCT screening. A total of 31,567 asymptomatic at-risk patients were initially screened using LDCT, and of these 27,456 patients were re-screened 7 – 18 months later. Thirteen per cent (4186) of patients were flagged up by baseline CT scans as positive
145 and required follow-up, and of these 484 later received a positive lung cancer diagnosis, with 412 patients presenting with early stage I lung cancer. The 10-year survival rate for these patients was found to be 88% [30]. Although these results are promising, they should be interpreted cautiously
150 because the study was not randomised and therefore may have been susceptible to bias.

Several trials have also looked at the effect of screening using sputum cytology, chest radiography or LDCT on mortality rates (reviewed in [31]), and a feasibility study
155 comparing LDCT with chest X-ray screening for lung cancer has successfully led to the implementation of the National Cancer Institute-funded National Lung Screening Trial (NLST) in the US. The NLST is a multi-centre randomised trial comparing LDCT and chest radiography as screening
160 tools for the early detection of lung cancer, and final results including mortality data are expected in 2010 [32].

2. Autoimmunity in cancer

165 A key feature of the human immune system is the ability to recognise self from non-self, and upon introduction of foreign matter such as virus particles a complex immune response is initiated. Part of this humoral response involves the production of antibodies that specifically target the foreign
170 matter. In some humans the immune system loses the ability to distinguish self from non-self, which can lead to a self-immunisation response, and in this instance antibodies are raised against the autologous host; these antibodies are commonly referred to as autoantibodies. The generation of
175 autoantibodies is usually indicative of an autoimmune disorder, such as rheumatoid arthritis, systemic lupus erythematous, Sjögren's syndrome, scleroderma and other related diseases [33].

Autoimmune responses can also be elicited by cancer
179 patients and this has been demonstrated through the

identification of autoantibodies against several intracellular 180 and surface antigens, often referred to as tumour-associated antigens, in patients with various types of cancer [34]. The mechanism behind the production of these auto-antibodies in cancer patients is still not clearly understood, but although circumstantial, evidence inferred from several 185 studies suggests that most autoantibody targets are cellular proteins whose aberrant expression and/or post-translational modifications have led to tumorigenesis [35].

2.1 Detecting autoantibodies *in vitro* 190
There are several methods now used for the detection of autoantibodies in the sera of cancer patients at various disease stages, and they include enzyme-linked immuno-sorbent assays (ELISA), proteomic methods such as phage display technologies and protein microarrays, agglutination 195 assays, immunoblotting, flow cytometry, immunoprecipitation and immunoflorescence, with the most popular and reproducible method of choice for autoantibody detection being ELISA. In a standard autoantibody ELISA micro-titre, 96-well plates are coated with recombinant tumour-associated 200 antigens, which are often produced in *Escherichia coli*. The patient's serum is incubated with these antigens on the plate, and autoantibodies present within the sera, specific for the antigens under investigation, bind to the antigen and are detected using anti-human immunoglobulin enzyme-tagged 205 antibodies and colorimetric measurement at a predefined end point. Following correction for nonspecific binding, results are compared with a set of normal control sera (ideally age and sex matched) and a cut-off value is determined above which a sample is considered positive. Statistically, the 210 most common determination of a positive autoantibody result by ELISA is achieved by using the 95 or 99% exact confidence interval, which means that a positive cancer response is a signal either 2 or 3 standard deviations above the mean signal of the healthy donor sera control group [36]. 215

The generation of autoantibody signatures for cancer, using other proteomic technologies such as protein microarrays, has also been proposed for the detection of lung cancer [37], and is reviewed in Imafuku *et al.* [38] and Shoshan and Admon [39]. The ultimate aim of all these lung cancer 220 detection techniques is to provide a test that has both high sensitivity and specificity.

3. Identification of tumour-associated antigens in lung cancer 225

The heterogeneity of lung cancer suggests that probably more than one biomarker will be needed for early cancer detection. The introduction of serological analysis of recombinant cDNA expression libraries (SEREX) has resulted 230 in the identification of a wide array of tumour-associated antigens eliciting B-cell responses in cancer patients. The technique involves screening cancer tissue complementary DNA libraries with autoantibodies present in patient sera. 234

235 Several SEREX studies have been carried out both in NSCLC and in SCLC [40-43] that have been able to identify several tumour-associated antigens such as CAGE-1 [42], XAGE-1b and NY-ESO-1 (CTAG-1) [43]. Although useful, SEREX has its disadvantages, the main one being the patient-

240 specific nature of a large proportion of the tumour-associated antigens detected [44]. Other techniques that can be used to identify tumour-associated antigens include proteomic screening using two-dimensional electrophoresis [45] and immunoscreening of lambda phage-based cDNA expression libraries with patient

245 sera [46]. Krause and colleagues have recently developed a new technique called seroGRID, which incorporates both SEREX and phage display. The technique allows detection of specific tumour-associated antigens using high-throughput screening of multiple SEREX-derived antigens that are

250 displayed on lambda phages against various patient and control sera [46]. This technique overcomes the aforementioned disadvantage of SEREX by allowing identification of antigens that are expressed in several cancer patients. In addition, by probing with healthy control sera, truly specific tumour

255 antigens can be distinguished. Alternatives to seroGRID, which combine phage display technologies with protein microarrays, have also been produced. These provide a platform technology for identification of new biomarkers as well as a method for validating such antigens using a large number of sera [37].

260 The classification of individual antigens is dependent on their exhibited patterns in normal and malignant tissues and several classes of tumour-associated antigen exist, which include mutated antigens, overexpressed antigens, differentiation antigens and miscellaneous antigens that may be regarded as

265 tumour-associated because of either aberrant changes to the protein itself, such as post-translational modifications, or altered behaviour of tumour cells leading to cellular dislocation of antigens and antigen release due to aberrant apoptosis.

270 ### 3.1 Mutated tumour-associated antigens
Probably the most well studied and understood auto-antibody response in cancer is to the tumour suppressor protein p53. Autoantibodies to p53 in cancer were first described by Crawford and colleagues in 1982, who demonstrated p53

275 autoantibody presence in 9% of breast cancer patients [47].

p53 is a key regulator of cell proliferation [48], and during cellular stress p53 can arrest cell cycle progression with two main outcomes, either the induction of apoptosis or DNA repair [49]. The p53 protein is encoded by the *p53* tumour

280 suppressor gene, and missense mutations in the coding region of the gene are found to be associated with most types of human cancer [50]. In lung cancer it is hypothesised that these mutations are a result of etiological exposure to carcinogens contained within tobacco smoke. Benzo[α]

285 pyrene diol epoxide (BPDE), a polycyclic aromatic hydro-carbon found in tobacco smoke, has been shown to form preferentially DNA adducts along exons of the *p53* gene in codons 157, 248 and 273; all of which are major mutational

289 hot spots in human lung cancer [51].

290 Missense point mutations in the *p53* gene ultimately lead to the production of mutant p53 protein with > 90% of the observed mutations being in the DNA binding domain [49]. The conformational changes that occur can stabilise the p53 protein, leading to its accumulation within tumour cells [52],

295 and this may provide a logical reason for the humoral immune response observed in some cancer patients. Interestingly, the autoantibodies against mutated p53 are raised against the immunodominant regions at the carboxyl and amino terminals of the protein [48], which is not in the mutational

300 hot spot regions as would be expected. This may provide an explanation as to why p53 mutations are observed in 90% of SCLCs and 50% of NSCLCs [53], but only ~ 7 – 27% of these patients have corresponding p53 autoantibodies [49]. Using immunoblotting, Winter and

305 colleagues demonstrated the absence of autoantibodies to p53 in the serum of patients with both frameshift and stop codon mutations (n = 10), and autoantibodies to p53 in only 13% (6 out of 46) of patients with point mutations [54]. This suggests that the humoral immune

310 response to p53 is dependent not only on the presence of point mutations within the gene, but also on the degree of overexpression and protein localisation [50], of which both are ultimately governed by p53 stability.

315 ### 3.2 Overexpressed tumour-associated antigens
The overexpression of a protein by tumours cells may provide a logical reason for the observed humoral immune response in some cancer patients, especially if the level of expression far exceeds the normal protein baseline

320 expression. A relationship between expressed immuno-genic antigens and amplified genes in SCLC was first demonstrated by Brass and colleagues, who showed a correlation between the level of gene amplification and overexpression of a gene product and the subsequent

325 production of autoantibodies [55].

The amplification of cellular oncogenes is an important mechanism of altered gene expression in cancers [40], and a common feature of lung cancer is the amplification of the *myc* proto-oncogene family, which includes c-*myc*, l-*myc* and

330 n-*myc* in both NSCLC and SCLC [56]. The *myc* genes encode nuclear phosphoproteins, which all have several motifs commonly associated with transcription factors [57]. The amplification of *myc* genes is commonly observed in lung cancer [58] and results in overexpression of myc proteins,

335 leading to rapid proliferation and loss of terminal differentiation. Autoantibodies to c-myc and l-myc have been described in 10 – 13% and 11 – 13% of lung cancers, respectively [35,36,57,59,60].

Brass and colleagues found that the oncogene encoding the translation initiation factor eIF-4gamma is amplified in

340 squamous cell lung cancer and is associated with autologous antibodies against the protein [40]; however studies into the frequency of eIF-4gamma autoantibodies in a cohort of

344 patients have yet to be established.

3.3 Differentiation tumour-associated antigens

Tumour-associated antigens that display altered expression patterns are often referred to as differentiation antigens, and like mutated and overexpressed antigens can elicit a humoral immune response in cancer patients. Recoverin is an N-myristoylated calcium-binding protein [61], which acts as a calcium-dependent modulator of rhodopsin phosphorylation in photoreceptor cells [62]. In healthy individuals it is normally restricted to the retina; however, it has paraneoplastic properties and can be aberrantly expressed in tumours outside the nervous system [63]. Expression of recoverin outside the nervous system triggers an immune response, and autoantibodies are raised against the protein, which targets not only the tumour expressed recoverin, but also normal retinal recoverin, which can cause cancer-associated retinopathy (CAR) in some patients [64]. Bazhin and colleagues demonstrated overexpression of recoverin in 68% of SCLC (30 out of 44) and 85% of NSCLC (34 out of 40) patients, and using immunoblotting they detected autoantibodies in 15 and 20% of the NSCLC and SCLC patients, respectively [62].

Another differentiation antigen expressed in lung cancer is NY-ESO-1. NY-ESO-1 is a member of a group of proteins known as the cancer/testis antigens, whose normal expression is restricted to male germ cells within the testis [65]. NY-ESO-1 is highly immunogenic and can elicit both cellular and humoral responses [66], and autoantibody frequencies in lung cancer have been reported to range from 4 to 23% [36,66,67]. Interestingly, NY-ESO-1 appears to be more associated with NSCLCs than SCLCs, although larger sample numbers are needed to confirm this [36].

3.4 Miscellaneous tumour-associated antigens

Some tumour-associated antigens demonstrate a mixture of overexpression, altered expression and aberrant post-translation modifications in cancer, which can lead to antigens becoming extremely immunogenic. Altered expression and aberrant glycosylation of annexin I and annexin II have been reported in lung cancer. Both antigens are members of the annexin multi-gene family of Ca^{2+}/lipid-binding proteins [68], most of which are intracellular proteins and sometimes comprise > 2% of total cellular protein weight [69]. All mammalian tissues examined for the presence of annexins express representatives of the annexin family, however no single cell type has been shown to express the entire family [70].

Annexins are generally associated with the inner cytoplasmic face of the plasma membrane, but have been found to be expressed on the surface of tumour cell types [69]; in addition, aberrant post-translational modifications have been reported [34]. The altered expression of these proteins along with aberrant glycosylation could elicit an immune response in cancer patients. Brichory and colleagues were able to demonstrate the presence of circulating IgG1 and IgM autoantibodies to annexin I and/or annexin II in 60% of patients with lung cancer (18 out of 30). The observed reactivity seen in these lung cancer patients was not limited to advanced stages, and 51% of patients with stage I cancer expressed autoantibodies [34]. In addition, the role of N-glycosylation in annexin immunogenecity was examined using de-glycosylated annexin I, and it was found to be dependent on N-glycosylation; however, this was not restricted to cancer cells or to immunoreactive patients as sera from immunoreactive patients also reacted with annexin I and II from normal lung. Based on this, Brichory and colleagues concluded that the change in levels and cellular distributions of annexins I and II in lung cancer patients were responsible for the levels of autoantibodies detected [34].

MUC1 is a membrane-bound glycoprotein that is both overexpressed and aberrantly glycosylated in cancer [71]. A recent study has demonstrated that a small section of this protein (corresponding to 45 amino acids of the extra-cellular domain) is sufficient to stimulate cell proliferation and chemotherapeutic resistance in MUC1-negative cells, suggesting oncogenic activity in this region [72]. Autoantibodies to MUC1 have been described in lung cancer [36], and it has been hypothesised that the presence of these antibodies could act as a positive prognostic factor in some individuals [73].

4. Autoantibodies for lung cancer screening and diagnosis

The presence of autoantibodies against tumour-associated antigens in the serum of lung cancer patients may have diagnostic potential, however, owing to the heterogeneous nature of cancer, it is unlikely that detection of autoantibodies to a single tumour-associated antigen would suffice as a diagnostic tool, as variation of individual antigen expression between patients is likely. However, the incorporation of several antigens into a panel-based assay could provide a highly specific and sensitive detection tool.

The detection of autoantibodies in 75% of 104 patients with lung cancer has recently been demonstrated using an ELISA-based panel of seven tumour-associated antigens (CAGE, GBU4-5, HER2, p53, c-myc, NY-ESO-1 and MUC1) with specificity for cancer of 92% (4 out of 50) [36]. A similar study carried out by Zhang and colleagues also detected autoantibodies in 68% of 56 lung cancer patients to 7 antigens (c-myc, p53, cyclin B1, p62, Koc, IMP1 and survivin) [35]. Both studies highlight the additive effect of using more than one antigen as individual autoantibody detection rates ranged only from 12% for c-myc and p53 up to 35% for MUC1 [36].

A recent study carried out by Chen and colleagues using phage display combined with protein microarrays accurately predicted cancer in 85% (64 out of 75) of patients with lung adenocarcinoma with a specificity of 86% [37]. In this study, a high-density phage-peptide microarray was developed by biopanning a phage display library derived from lung

Table 2. Tumour-associated antigen ELISA panels and their sensitivity in detecting lung cancer.

Patient cohort	Antigens used	Sensitivity (%)	ELISA cutoff (n)	Ref.
56 lung cancer patients	Recombinant c-myc, p53, cyclin B1, p62, Koc and IMP1	68	> Mean + 2 s.d. (82)	[35]
82 NSCLC and 22 SCLC patients	MUC1 peptide. Recombinant CAGE, GBU4-5, HER2, p53, c-myc and NY-ESO-1	76	> Mean + 2 s.d. CAGE and GBU4-5 (50) > Mean + 3 s.d. for other antigens (50)	[36]
67 lung cancer patients	Human collagen types I – V	43	> Mean + 2 s.d. (50)	[87]
120 squamous lung cancers (stages I – III)	Recombinant Tim and MnSOD	20	> Mean + 3 s.d. (40)	[45]
56 lung cancer patients (sera collection at time of diagnosis)	Recombinant c-myc, p53 and survivin	32	> Mean + 3 s.d. (82)	[57]
24 lung cancer patients (primary operable disease)	Recombinant NY-ESO-1, MAGE-1, MAGE-3, melan-A, tyrosinase, SSX2 and Carbonic anhydrase	8	> Mean + 3 s.d. (70)	[67]
28 NSCLC and 9 SCLC patients (stages I – IV)	Recombinant livin and survivin	71	> Mean + 2 s.d. (7)	[95]
84 lung cancer patients	Recombinant p62 and Koc	20	> Mean + 3 s.d. (82)	[96]

The cutoff describes how autoantibody positivity was determined using a minimum of 95% confidence intervals.
ELISA: Enzyme-linked immunosorbent assay; MUC1: Mucin 1; n: Number of normal sera analysed to generate cutoff; NSCLC: Non-small cell lung carcinoma; SCLC: Small cell lung carcinoma; s.d.: Standard deviation.

cancer tissue pools, which was then used to assess whether autoantibody signatures could be used to detect cancer. Using a training set of 125 serum samples (75 cancers and 50 controls), autoantibodies were ranked based on their classification accuracy and 22 discriminating peptides were then selected for the final test. Some of these peptides were found to encode for a cancer-associated protein, ubiquilin 1, and autoantibodies for 2 isoforms of this protein were significantly higher in cancer sera compared with the controls [37].

A smaller study carried out by Qiu and colleagues evaluated the use of natural protein micoarrays (from the A549 human lung adenocarcinoma cell line) to measure autoantibody response in cancer patients. Immunoglobulin reactivity from 18 lung cancers and 15 controls was measured and 63 of the 1840 A549 cell lysate fractions showed reproducible reactivity to lung cancer patients relative to the controls [74]. Although the authors did not go on to identify the reactive antigens present in the positive fractions, the study highlights the possibility of using cancer cell lines to isolate natural tumour-associated antigens as an alternative to recombinant antigens, which may lack cancer-specific epitopes that have arisen from aberrant post-translational modifications. Several other antigen panel assays have been reported and these are summarised in **Table 2**.

4.1 Detection of autoantibodies before clinical diagnosis

The production of autoantibodies has also been reported months and even years before clinical diagnosis of lung cancer. Early evidence to support the theory that autoantibodies are present before clinical diagnosis of the disease arises in cancer patients suffering from paraneoplastic neurologic disorders (PND). Gene expression of neural antigens usually restricted to the nervous system by tumour cells can often lead to an antitumour immune response [75] and generation of antionconeural antibodies that react with both the cancer-expressed protein and the normal protein within the nervous system, leading to PND in patients [76]. One such disorder, known as Lambert-Eaton myasthenic syndrome (LEMS), is commonly associated with SCLC patients [77], and a common manifestation of SCLC patients with LEMS is the presence of IgG antibodies to P/Q-type voltage-gated calcium channels (VGCC), which are responsible for the muscle weakness associated with the disease. Studies have shown that the onset of LEMS can precede SCLC diagnosis by up to 2 years [78], making VGCC autoantibodies an interesting candidate marker of early pre-invasive SCLC. In addition to VGCC autoantibodies, anti-glial nuclear antibodies have been detected in the serum of patients with LEMS and SCLC, and Sabater and colleagues found that these anti-glial nuclear antibodies are raised against an antigen known as SOX-1 and have reported autoantibodies in 64% of patients with LEMS and SCLC [79].

Autoantibodies against p53 have previously been detected before cancer diagnosis in smokers with chronic obstructive pulmonary disease (COPD) [80] and in patients with asbestosis [52] and idiopathic pulmonary fibrosis [53].

511 An early study by Trivers and colleagues recruited 67 patients with COPD who were tested for the presence of p53 autoantibodies; 23 of the COPD patients went on to develop lung cancer, and of these 22% showed detectable levels of
515 serum p53 autoantibodies, and 80% of those patients with p53 autoantibodies showed detectable levels before the cancer diagnosis. In addition, no autoantibodies were detected in the 44 COPD patients who did not develop cancer during the 5-year study [80]. These findings are not
520 restricted to p53, and autoantibodies to survivin in the sera of 2 lung cancer patients at 18 and 12 months before clinical manifestation of the disease have been described [81], as well as autoantibodies to 14-3-3θ, protein gene product 9.5, annexin I [82] and CENP-B [83].

525 In addition to autoantibody responses to single tumour-associated antigens, Zhong and colleagues have successfully detected autoantibodies to a miniarray of tumour-associated antigens in pre-diagnostic sera taken from NSCLC patients enrolled on the Mayo Clinic Lung Screenng Trial. T7-phage
530 NSCLC cDNA libraries were screened with patient plasma and 212 immunogenic phage-expressed proteins were identified, which were ranked statistically for their individual reactivity against a training set of sera comprising 23 stage I NSCLC samples and 23 risk-matched controls. Five out
535 of the 212 immunoreactive phage-expressed proteins were selected by reactivity and used to screen 102 samples from the Mayo Clinic Lung Screening Trial, which comprised 56 non-cancer samples, 6 prevalence samples and 40 cancer samples, which were drawn 1 – 5 years before
540 detection by incidence screening. The array accurately predicted all 6 prevalence cancers and 80% (32 out of 40) of the pre-diagnostic samples, with an overall specificity of 87.5% [84].

These findings suggest that autoantibody detection would
545 be a useful screening tool in high-risk patients, allowing detection of lung cancer at its earliest stage and ultimately leading to early intervention of the disease, which may impact patient prognosis.

5. Autoantibodies to monitor disease and
550 predict clinical outcome in lung cancer

The measurement of fluctuating tumour-associated antigen titres is now used to monitor disease regression and progression
555 in cancer patients during therapy. Tumour-associated antigens used for this purpose are commonly referred to as tumour markers and classic examples include carcino-embryonic antigen (CEA), CA15.3 (MUC1), cytokeratin 18 (tissue-specific antigen assay), cytokeratin 19 (CYFRA 21-1 assay) and
560 cytokeratins 8, 18 and 19 (tissue polypeptide antigen assay). Unfortunately, there are limited numbers of examples correlating autoantibody titres with clinical outcome of cancer; however, several studies have highlighted the potential use of auto-antbodies in disease monitoring, particularly regarding
565 autoantibodies raised against overexpressed antigens.

Zalcman and colleagues demonstrated fluctuations in 566 p53 autoantibody titres during lung cancer patient therapy. Sixteen patients with and 16 patients without detectable p53 autoantibodies were selected from 167 screened lung cancer patients and monitored for p53 570 autoantibodies over a 30-month treatment period. Twelve out of the 16 who were initially positive for the p53 autoantibody showed a decrease in antibody levels by > 50%, and 8 of the 16 underwent a complete response to therapy [85]. In another study, reduction in p53 575 autoantibody titre was observed in 22 out of 38 patients who underwent surgical resection, however follow-up of the patients carried out over a 9-year period showed no relationship between titre levels and clinical outcome or disease course [86]. 580

Autoantibodies to collagen types I, II, III, IV and V have been reported in the sera of patients with lung cancer [87], with sensitivity ranging from 28% for type I collagen to 11.9% for type V, along with a relationship between autoantibody levels and patient outcome, whereby increased 585 titres of collagen type I and IV autoantibodies conveyed a favourable outcome including increased progression-free survival, increased survival and increased duration of favourable response, and conversely increased collagen type V conveyed a less favourable outcome by correlating with 590 decreased progression-free survival [87]. NY-ESO-1 auto-antibody titres have also been shown to fluctuate in patients with both progressive disease and in therapy-induced regression patients with various cancer types [88].

595

6. Autoantibodies and lung cancer prognosis

A small number of tumour-associated antigens that elicit an autoantibody response show correlation with lung cancer prognosis (**Table 3**). Interestingly, the presence of some 600 autoantibodies in lung cancer correlates with a favourable prognosis, and examples include the presence of antineural and antinuclear autoantibodies and MUC1-specific autoantibodies in both NSCLC and SCLC [73,89-91].

Graus and colleagues demonstrated Hu autoantibodies in 605 16% (27 out of 170) of patients with SCLC who had undergone treatment, and found there was statistically significant association between the presence of Hu auto-antibodies and complete response to therapy (55.6 versus 19.6%), which in turn increased patient survival from 610 10.2 to 14.9 months [90].

Maddison and colleagues investigated differences in survival time in patients with SCLC and LEMS who had raised titres of anti-P/Q-type VGCC antibodies from a matched group of patients with SCLC only. Interestingly, they found 615 a shorter median survival time of 10 months from time of diagnosis in patients with SCLC compared with 17.3 months in patients with SCLC and LEMS [77]. By contrast, poor prognosis has been associated with the presence of p53 autoantibodies (**Table 4**). 620

Table 3. Correlation of clinco-pathological variables and survival with frequency of autoantibodies to tumour-associated antigens expressed in lung cancer.

Antigen	Methodology	Autoantibody frequency	ELISA cutoff (n)	Comment	Ref.
p53	Various	7 – 27%	Various	See **Table 4**	
Recoverin (recombinant)	WB	15% SCLC (15/99)	N/A	Increased frequency of CAR	[62]
		20% NSCLC (9/44)			
		10% SCLC (5/50)	N/A		[64]
Hsp40 (recombinant)	ELISA	50% (25/50)	> Mean + 2 s.d. (130)	Not reported	[97]
Alpha-enolase (cultured A549 cell line)	2D PAGE/WB	60% (3/5)	N/A	Not reported	[98]
Alpha-enolase (recombinant)	ELISA	28% NSCLC (26/94)	> Mean + 3 s.d. (60)		[99]
		0% SCLC (0/15)			
NY-ESO-1 (recombinant)	ELISA	20% (35/175)	> Mean + 3 s.d. (51)	Associated with histological grade	[66]
		18% (19/104)	> Mean + 3 s.d. (50)		[36]
		4% (1/24)	> Mean + 3 s.d. (70)		[67]
Annexin I (cultured A549 cell line and solid tumour)	2D PAGE/WB	30% (12/30)	N/A	Not reported	[34]
Annexin II (cultured A549 cell line and solid tumour)	2D PAGE/WB	33% (18/54)	N/A	Not reported	[34]
c-myc	WB	13% (9/68)	N/A	No correlation between histological stage, smoking history and treatment in anti-c-myc positive and anti-c-myc negative patients	[60]
Protein gene product 9.5 (cultured A549 cell line and solid tumour)	2D PAGE/WB	14% (9/64)	N/A	Not reported	[100]
Hu	WB	16% SCLC (7/44)	N/A	Associated with limited stage, complete response to therapy and longer survival	[89]
		16% (32/196)			[90]
SOX1 (recombinant phage)	λ-phage plaque assay	28% SCLC (25/90)	N/A	Associated with younger age, lower LDH levels and better response to therapy	[101]
ZIC 2 (recombinant phage)	λ-phage plaque assay	28% SCLC (25/90)	N/A		[101]
Survivin (recombinant)	ELISA	22% (11/51)	> Mean + 3 s.d. (300)	Not reported	[81]
		20% (37/189)	> Mean + 3 s.d. (82)		[57]
		58% (18/31)	> Mean + 2 s.d.D (7)		[95]

The cutoff describes how autoantibody positivity was determined using a minimum of 95% confidence intervals.

CAR: Cancer-associated retinopathy; ELISA: Enzyme-linked immunosorbent assay; Hsp: Heat-shock protein; LDH: Lactate dehydrogenase; n: Number of normal sera used to generate mean; N/A: Not applicable; NSCLC: Non-small cell lung carcinoma; SCLC: Small cell lung carcinoma; s.d.: Standard deviation; WB: Western blotting; 2D PAGE: 2-Dimensional-polyacrylamide gel electrophoresis.

Table 4. Frequency of p53-specific autoantibodies in the sera of patients with lung cancer and their correlation with patient prognosis.

Frequency of autoantibodies	Methodology	Comment	Ref.
27% (18/67)	ELISA	Better survival after radiotherapy in NSCLC	[102]
23% (19/84)	ELISA	Correlation with shorter survival in NSCLC, particularly in squamous cell carcinomas	[103]
NSCLC 8% (9/111) SCLC 7% (1/14)	WB	Autoantibody presence associated with malignant pleural effusion and independently shows poor prognosis	[104]
21% (20/97)	ELISA	Poor prognosis for limited stage	[105]
13% (17/134)	ELISA	Correlation with shorter survival in NSCLC only	[106]
20% (38/188)	ELISA	Association with histological type and stage	[86]
19% (25/133)	ELISA	Association with stage	[107]

ELISA: Enzyme-linked immunosorbent assay; NSCLC: Non-small cell lung carcinoma; SCLC: Small cell lung carcinoma; WB: Western blotting.

7. Conclusions

The presence of circulating autoantibodies to tumour-associated antigens in lung cancer has been clearly demonstrated in several studies reviewed within this article, both individually and when combined into a panel-based ELISA antigen assay, as well as in protein microarray studies; and their use in diagnosis, prognosis and monitoring clinical outcome has been investigated.

There are limited results obtained so far for the use of autoantibodies as markers of prognosis and for monitoring clinical outcome, however the results generated are interesting and warrant continued investigation. It is believed that the production of autoantibodies against tumour-associated antigens occurs early in cancer development, and support for this arises from:

- detection of circulating autoantibodies in the sera of patients predating the time of clinical diagnosis
- the presence of symptomatic autoimmune PNDs such as LEMS years before lung cancer diagnosis is made
- autoantibodies present in the sera of lung cancer patients are often of the IgG class, indicating a mature secondary response.

In conclusion, it is likely that low levels of tumour-associated antigens in early disease elicit an immune response and the measurement of circulating autoantibodies raised against these antigens could provide clinicians with a simple, non-invasive and cost-effective opportunity to detect *in vivo* amplification of the early carcinogenic signal.

8. Expert opinion

The detection of advanced lung cancer is a key factor contributing towards the poor prognosis for most patients who are diagnosed, because more often than not curative surgical intervention is no longer an option due to tumour size and presence of metastases. At present, although smoking is a known risk factor for the development of lung cancer, there is no screening test available to identify early disease when the chances of survival are higher.

There is therefore an urgent need to develop a screening tool that is sensitive, specific, simple and cost-effective for the earlier detection of lung cancer at a stage where successful treatment can be administered, particularly in high-risk patient groups. At present, the main focus of interest regarding early diagnosis of lung cancer is the use of LDCT. This has been shown to detect more pulmonary nodules than standard chest radiography; however it is important to realise that LDCT is extremely costly and demonstrates a higher rate of false-positives when compared with chest radiography [92], which can ultimately lead to unnecessary invasive diagnostic procedures and undue stress for the patients. In addition the lungs are very sensitive organs, particularly to radiation, so radiation-induced lung cancer risk caused by regular screening using LDCT would need to be thoroughly examined, particularly in smokers, as radiation damage and smoking damage have been shown to interact synergistically [93]. The detection of autoantibodies to tumour-associated antigens in patient sera could provide an alternative approach to screening for lung cancer as a blood test is relatively non-invasive, cost-effective and, most importantly, has no long-term side effects, ultimately allowing more frequent sampling compared with LDCT.

Reviewing the current literature regarding the presence of autoantibodies in lung cancer has revealed that measurement of autoantibodies to a combination of tumour-associated antigens in a panel-based assay conveys a far greater sensitivity and specificity when compared with autoantibody measurements of individual antigens, indicating the heterogeneous nature of cancer between patients. Therefore, future research needs to focus on development of a panel-based assay for the detection of multiple autoantibodies raised against key antigens involved in lung cancer. This will involve optimisation of a panel of tumour-associated antigens to achieve maximum sensitivity and, more importantly, specificity.

697 An optimal panel assay for lung cancer detection needs to detect autoantibodies raised against lung cancer-specific antigens (NSCLC and SCLC) along with autoantibodies

700 against antigens commonly expressed in cancer ('universal autoantibodies') such as cell cycle proteins p53 and survivin, and the development of such a panel-based assay needs to be structured and follow a logical progression similar to that described by Sullivan Pepe and colleagues for all potential

705 biomarkers [94]. The key factor for the successful implementation of an autoantibody panel-based detection assay in the clinical setting will be its ability to detect possible underlying lung cancers in the normal population. This will involve further research of autoantibody measurements in carefully

710 controlled studies (age, sex and smoking history) where pre-diagnostic samples and diagnostic samples taken from patients with newly diagnosed early lung cancer, as well as patients with benign lung disease, autoimmune diseases and individuals at an increased risk of developing lung cancer,

715 are compared to produce a set of specific and sensitive candidate biomarkers for clinical use. Continued development of methodology and comparison studies should be carried out to determine the most suitable technique to implement,

719 which, as well as being specific and sensitive must be reproducible, for example, a simple panel-based ELISA assay or a more complex protein microarray kit. 720

It is also feasible that LDCT/chest radiography and autoantibody panel assays could be used in conjunction, whereby autoantibodies are first measured in high-risk patients, and those demonstrating autoantibody positivity are followed 725 up using imaging modalities. Those that are autoantibody positive but imaging negative may require further intensive follow-up. Future retrospective and prospective trials in high-risk groups may accurately identify the true sensitivity and specificity of such tests. 730

Investigations into the use of autoantibodies as diagnostic markers are still in the research phase, however the growing interest in their potential as early diagnostic markers should eventually progress to the development of a diagnostic test that can be used in the clinical setting, 735 alongside other diagnostic techniques, with the ultimate goal of saving lives.

Declaration of interest
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Affiliation

Kelly M Woodard PhD, Research Student & Caroline J Chapman[†] PhD
[†]Author for correspondence
The University of Nottingham,
Division of Breast Surgery,
Clinical Sciences Building,
Nottingham City Hospital,
Nottingham NG5 1PB, UK
Tel: +44 0 1158231825; Fax: +44 0 1158231877;
E-mail: caroline.chapman@nottingham.ac.uk