

# Blood based biomarkers beyond genomics for lung cancer screening

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**Abstract:** While there is considerable interest at the present time in the development of so-called liquid biopsy approaches for cancer detection based notably on circulating tumor DNA, there are other types of potential biomarkers that show promise for lung cancer screening and early detection. Here we review approaches and some of the promising markers based on proteomics, metabolomics and the immune response to tumor antigens in the form of autoantibodies.

**Keywords:** Biomarkers; proteins; metabolites; autoantibodies

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## Introduction

A major contributing factor to high mortality in lung cancer is that most cases are diagnosed at advanced stages with limited benefits from currently available therapies, hence the interest in implementing screening strategies to detect lung cancer at an early stage. The findings from the National Lung Screening Trial (NLST) study, which demonstrated a 20% reduction in lung cancer mortality with low-dose CT (LDCT) screening of subjects aged 55–74 years with a 30-pack-year smoking history, have generated interest in lung cancer screening (1). Based on these findings the US Preventive Services Task Force recommended LDCT screening of subjects that meet the NLST criteria. However the adoption of lung cancer screening has been quite modest and a cautious approach to screening has been advocated (2). Concerns about the high percentage of false positives and potential health hazards resulting from exposure to radiation with repeat LDCTs as well as concern about the fact that a majority of subjects destined to be diagnosed with lung cancer are currently not eligible for screening have heightened the need to identify

subjects at risk for developing lung cancer that do not meet current eligibility criteria. Biomarkers offer the potential for precision medicine across the cancer continuum from risk assessment to early detection and tumor classification (3). For lung cancer in particular, approaches in which a biomarker based initial screen is followed by LDCT or in which a biomarker test is combined with LDCT have merit for early detection applications (4,5).

## Sources and types of biomarkers for lung cancer detection

In principle, biomarkers that may aid in identifying subjects that may benefit from LDCT or that complement LDCT such as for assessment of indeterminate nodules could be found in various biological fluids notably blood, urine, saliva or sputum (6–8). Exhaled breath has also been explored as a source of biomarkers (9–11) as well as airway brushing (12). Blood in particular, with its cellular, micro-particulate, and plasma constituents, represents a rich source of biomarkers.

Although considerable attention is focused on liquid biopsy approaches for lung cancer detection (13), it is

likely that no single marker or approach will be useful alone, hence the need to explore combinations of markers and approaches to determine their performance. This undertaking represents a daunting effort. There are thousands of publications in the literature pertaining to potential lung cancer biomarkers. Testing biomarker candidates singly or in combination with informative biospecimens relevant to the intended application is generally beyond the means of individual laboratories and requires a collaborative effort as currently being undertaken through the National Cancer Institute Early Detection Research Network. Here, we limit the review to circulating protein, metabolite and humoral immune response based biomarkers that have potential for screening and early detection of lung cancer.

### Autoantibodies

A humoral immune response in the form of autoantibodies to tumor antigens occurs early during tumor development. Identification of antigens that induce a selective autoantibody response in lung cancer has the potential to yield markers useful for early detection applications. A recent PubMed search of autoantibodies in lung cancer yielded 1,090 publications. A sampling of autoantibody-based biomarker combinations that have been published is provided in *Table 1*. A review of the literature found that the most commonly used detection method was enzyme-linked immunoassay (ELISA), applied in 31 out of 38 tests for single autoantibodies and in 33 out of 49 tests for multiple autoantibodies. Multiplex assays are exemplified in a panel of seven autoantibodies to tumor antigens which yielded improved sensitivity compared to individual autoantibodies or smaller panels (32).

Another study involved a panel of 25 serum autoantibodies associated with non-small cell lung cancer (NSCLC) that were tested in a protein microarray format containing the autoantigens using sera from 125 patients with NSCLC and 125 matched controls with a benign nodule (63). In the training data set the logistic regression c-index statistic was 0.691 and 0.490 in the test set. The relationship between the score and outcome was not statistically significant ( $P=0.460$ ). A meta-analysis of 31 articles with single autoantibodies and 39 with multiplex autoantibodies was recently done, supporting improved diagnostic accuracy with multiplex panels (64).

An ongoing study is intended to determine whether a panel of autoantibodies can identify subjects at high

risk for lung cancer, followed by X-ray and computed tomography (CT) scanning, resulting in reduced incidence of patients with late-stage lung cancer (III & IV) or unclassified presentation at diagnosis, compared to standard practice (65). The study involves 12,000 participants aged 50–75 who are at high risk for lung cancer in Scotland. The intervention is a seven-autoantibody test, followed by X-ray and CT for subjects with a positive result. The primary outcome is the difference, after 24 months, between the rates of patients with stage III, IV or unclassified lung cancer at diagnosis. Participants with a positive test result but for whom the CT scan does not lead to a lung cancer diagnosis will be offered follow-up CTs for 24 months. An initial chest X-ray will be used to determine the urgency and the need for intravenous contrast in the first screening CT. Participants who are found to have lung cancer will be followed to assess both time to diagnosis and stage of disease at diagnosis.

From a practical perspective a biomarker approach based on autoantibodies is appealing as antigenic targets could be printed or synthesized on a chip. Hybridization to detect autoantibody reactivity would require no more blood volume than a drop of blood. Additionally, with the availability of autoantibody marker panels, each having specificity to one of the common cancers, a chip could be produced that would allow screening across cancer types. However, at present, the definitive validation of the utility of autoantibody biomarkers for screening either alone or in combination with other markers has not been demonstrated.

### Protein based biomarker panels

A wide world of technologies is currently available for the discovery of protein markers as well as for the development of corresponding assays with applications to lung cancer (*Table 1*). Widely used approaches consist of multiple forms of mass spectrometry for both discovery and assay development and the use of antibody arrays for discovery and ELISA for assay development either singly or in multiplex formats.

At present time, several protein-based biomarkers are available in the clinic that are primarily used for diagnosis or for patient monitoring. These assays are particularly suited for subjects with symptoms or for imaging-based findings including stratification of indeterminate nodules. Guidelines for the management of subjects with nodules detected through CT screening are evolving. The National Comprehensive Cancer Network (NCCN)

**Table 1** Lung cancer blood based biomarker combinations reported

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Protein based biomarker combinations

ProGRP, CEA, SCC, and CYFRA21-1 (14)

MIC-1, Cyfra21-1, CA125 and CEA (15)

CEA, CA125, ProGRP, and MCP-1, IL6, IL10 (16)

LRP1, BGH3, COIA1, TETN, TSP1, ALDOA, GRP78, ISLR, FRIL, LG3BP, PRDX1, FIBA and GSLG1 124/123 71 44 LR (17)

Cadherin-1, CD30 ligand, endostatin, HSP90 $\alpha$ , LRIG3, MIP-4, pleiotrophin, PRKCI, RGM-C, SCF-sR, sL-selectin and YES (18)

CYFRA 21-1, NSE and CRP (19)

CRP, SAA and mucin 1 (20)

CEA, RBP, A1AT and SCC 49/48 77.8 75.4 CART (21)

CYFRA 21-1, NSE and CRP (22)

MIP-1 $\alpha$ , CEA, SCF, TNF-RI, IFN- $\gamma$  and TNF- $\alpha$  (23)

CYFRA 21-1, NSE and CRP (24)

$\beta$ 2-microglobulin, CEA, gastrin, CA125, NSE and sIL-6R (25)

CEA, gastrin and NSE (26)

Prolactin, TTR, THBS1, E-selectin, CCL5, MIF, PAI-1, erbB-2, CYFRA 21.1 and SAA (27)

A1AT, Cyfra 21-1, IGF1, RANTES and AFP (28)

CYFRA 21-1, CEA, CA-125 and CRP (29)

MMP-1, MMP-9, CYFRA 21-1, CRP, CEA, YKL-40 and CA-125 (30)

IL-6, IL-10, IL-1ra, sIL-2R $\alpha$ , SDF-1 $\alpha$ + $\beta$ , TNF- $\alpha$  and MIP-1 $\alpha$  (31)

Autoantibody based marker combinations

p53, CAGE, NY-ESO-1, GBU4-5, SOX2, MAGE A4 and Hu-D (32)

p53, PGP9.5, SOX2, GAGE7, GBU4-5, CAGE, and MAGEA1 (33,34)

Survivin, Cyclin B1, HCC1, and p53 (35)

BARD1 epitopes (36)

ECH1 and HNRNPA2B1 (37)

ANXA1 and FOXP3 (38)

14-3-3 $\zeta$ , c-Myc, MDM2, NPM1, p16, p53 and cyclin B1 (39)

MDM2, C-Myc (40)

keratin 8, type II, TTC14, Kruppel-like factor 8, BRAF, and tousel like kinase 1 (41)

p53, NY-ESO-1, CAGE, GBU4-5, annexin A1 and SOX2 (42,43)

p53, NY-ESO-1, CAGE, GBU4-5, SOX-2, HuD, MAGE A4,  $\alpha$ -enolase, p53 C, CK8, CK20 and Lmyc2 (30)

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**Table 1** (continued)**Table 1** (continued)

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Autoantibody based marker combinations

c-Myc, cyclin A, cyclin B1, cyclin D1, CDK2 and survivin (44)

IMPDH, PGAM, HSP70-9B, Ubiquilin, annexin A1 and annexin A2 (45)

paxillin, SEC15L2, BAC clone RP11-499F19, XRCC5 and MALAT1 (46)

Metabolites

Glucose lactate and phospholipids (47)

Lysophosphatidylcholines (48)

Glutamic acid, choline, threonine (49)

Glutamic acid, aspartic acid, xylose (50)

Phosphorylcholine, sphingosine (51)

Glycerol, lactic acid, tryptophan (52)

Benzaldehyde, urea, isoleucine, glycolic, phenylalanine (53)

Bilirubin (54)

Citric acid, choline, lysine (55)

Alanine, threonine, linoleic acid (56)

Alanine, valine, isoleucine (57)

Carnitine, propionylcarnitine, tyrosine, methionine, malic acid, histidine, 5-oxo-proline (58)

Mixed panels of markers

Proteins CEA, CYFRA21-1, CA125, and NY-ESO-1 autoantibody (59)

Proteins CEA, CYFRA21-1, ENO1 and ENO1 autoantibody (60)

Pro-surfactant B protein and diacetylspermine metabolite (61)

C-reactive protein (CRP), prolactin and hepatocyte growth factor (HGF), NY-ESO-1 autoantibody (62)

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provides guidelines that avoid invasive follow-up for small nodules with low probably of malignancy (NCCN.org). The guidelines incorporate recommendations based on the NLST, the International Early Lung Cancer Action Program protocols and the Fleischner Society guidelines. Reporting systems have been suggested that classify nodules observed with screening CTs based on the risk of malignancy which is linked to suggested follow-up [American College of Radiology. Lung CT Screening Reporting and Data System (Lung-RADS). Available at: <http://www.acr.org/Quality-Safety/Resources/LungRADS>] (66). A recent study encompassed 691 patients

with pulmonary lesions from three centers in China and was intended to develop and validate a nomogram model based on clinically available tumor markers to assess the likelihood of cancer for patients with pulmonary lesions (67). The predictive performance of the nomogram was measured by concordance index and calibrated with 1,000 bootstrap samples to reduce over-fitting. A multivariate logistic regression model identified tumor size, CEA, SCC, CYFRA21-1, pro-GRP, and HE4 as independent risk factors for lung cancer. The nomogram developed showed concordance index of 0.901 (95% CI: 0.842–0.960;  $P < 0.001$ ) for lung cancer in the training set and 0.713 (95% CI: 0.599–0.827;  $P < 0.001$ ) in the validation set.

A multitude of other studies have explored the contribution of existing protein lung cancer biomarkers as well as additional markers resulting from discovery studies. One group utilizing mass spectrometry developed a 13-protein blood-based classifier that differentiated malignant and benign nodules with high confidence (17). The group identified 371 protein candidates and developed a mass spectrometry based multiple reaction monitoring assay for each. The classifier was validated on an independent set of plasma samples with good performance. The source and the relationship of the tumor markers to tumor characteristics was not clear. In a subsequent study, an integrated model incorporating clinical risk factors and proteomic molecular markers was developed and its performance assessed on a subset of 222 lung nodules, between 8 and 20 mm in diameter (68). Whereas the protein markers were found to be most predictive, the integration of protein markers with clinical features was found to be superior. Rigorous independent validation of these markers is required before such a test is adopted.

### Metabolites as lung cancer biomarkers

Comprehensive metabolomic profiling using high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), encompassing a few thousand identified metabolites covering metabolic pathways relevant to health and disease has become well established. Metabolomic profiling offers a dynamic view of cell tissue and organismal function. Active effort is ongoing in the search for metabolomics biomarkers that have relevance to lung cancer detection (69) (Table 1).

Phospholipids are important constituents of the cell membrane. Phospholipid metabolic pathways are

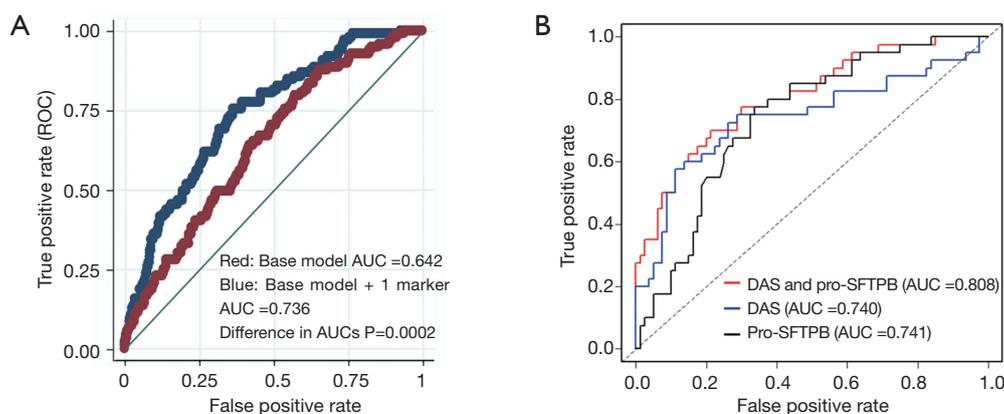
upregulated in lung cancer yielding distinct signatures (70). A blood based metabolomics study was conducted as part of lung cancer screening (71). Analysis encompassed 31 patients with screen detected lung cancer and a matched group of 92 healthy controls. Several metabolites in serum discriminated patients from controls. Most were downregulated in cancer samples, including amino acids, carboxylic acids and tocopherols. Benzaldehyde was the only compound found to be significantly upregulated. A follow-up study based on lipid profiling encompassed 100 patients with early stage lung cancer, including 31 screen-detected cases and a matched group of 300 healthy screened subjects (48). Downregulation of lysophosphatidylcholines (LPC18:2, LPC18:1 and LPC18:0) distinguished cases from controls. Other studies have uncovered a diversity of metabolites that distinguished cases from controls (Table 1) thus necessitating rigorous validation studies that address specific intended clinical application(s) for their validation.

### Modeling lung cancer risk based on subject's characteristics and biomarker profile

An intended goal of molecular testing is to identify subjects that would benefit from LDCT. There is not a currently recommended risk prediction model for lung cancer for general use. Several models based on subject's characteristics have been proposed (72–74). The addition of pulmonary function has been shown to improve risk prediction (75,76). Cotinine, a circulating metabolite of nicotine has been found to be associated with lung cancer risk (77), and may represent a more sensitive assessment of smoking intensity than self-reporting.

The contribution of inflammatory markers to lung cancer risk has been explored. Circulating levels of CRP, SAA, sTNFR2, and CXCL9/MIG were found to be associated with lung cancer risk in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (78). However, these markers did not improve lung cancer risk stratification beyond models based on subject characteristics. Cytokine profiling has revealed an increased risk of lung cancer associated with high levels of IL6 and IL8 (79). A causal role of fasting insulin and low-density lipoprotein cholesterol in lung cancer etiology, and for BMI in squamous cell and small cell carcinoma, has been suggested (80).

Vitamin status has been associated with risk of numerous diseases including cancer (81,82). A nested case-control study within the European Prospective Investigation into Cancer and Nutrition provided strong evidence that



**Figure 1** Pro-SFTPb and DAS as risk markers. (A) Receiver operator characteristic of a smoking based risk model applied to the Pan Canadian Lung Screening cohort and significantly improved performance of the combined base model together with pro-SFTPb; (B) additive performance of Diacetyl spermine, together with pro-SFTPb in a blinded validation study using pre-diagnostic samples from a high risk cohort.

increased vitamin B6 catabolism is independently associated with a higher risk of future lung cancer (83). A recent study of circulating folate, vitamin B6, and methionine found that subjects with higher circulating concentrations of vitamin B6 and folate had a modestly decreased risk of lung cancer (82). The kynurenine/tryptophan ratio has also been found to be associated with increased risk of lung cancer (84). A study that involved a prospective cohort of 395,875 participants in Taiwan was conducted to improve risk prediction models for lung cancer by incorporating biomarkers (85). Age, gender, smoking pack-years, family history of lung cancer, personal cancer history, BMI, lung function, and serum biomarkers were included as variables. The area under the curve (AUC) in overall population was 0.851 (95% CI: 0.840–0.862), with never smokers 0.806 (95% CI: 0.790–0.819), light smokers 0.847 (95% CI: 0.824–0.871), and heavy smokers 0.732 (95% CI: 0.708–0.752). By integrating risk factors together with CEA and AFP for light smokers, and lung function testing, AFP, and CEA for light and never smokers, cancer risks as high as those for heavy smokers could be identified. The risk model for heavy smokers allowed stratification of heavy smokers into subgroups with distinct risks.

Few studies have assessed the contribution of biomarkers based on rigorous validation studies using lung cancer screening cohorts. Proteomic studies have led to the identification of several candidate markers associated with the development of lung cancer including pro-surfactant protein B (pro-SFTPb), a target of NKX2-1, a lineage survival oncogene in lung cancer (86). Multivariable logistic regression models were used to evaluate the predictive

ability of pro-SFTPb among subjects undergoing LDCT screening. The AUC values of the full model with and without pro-SFTPb were 0.741 (95% CI: 0.696 to 0.783) and 0.669 (95% CI: 0.620 to 0.717), respectively (difference in AUC,  $P=0.0002$ ) (87). (Figure 1A) In a related study, a mass spectrometry method designed to profile a wide range of metabolites was applied to pre-diagnostic serum samples from a high-risk cohort that encompassed 100 subjects who subsequently developed NSCLC and 199 matched controls. A separate aliquot was used to quantify levels of pro-SFTPb (61). N1, N12-diacetylspermine was identified as a metabolite with significant increase in concentration in samples from cases compared to controls. A validation study of DAS and pro-SFTPb was performed using an independent set of pre-diagnostic sera from 108 patients with NSCLC and 216 matched controls. DAS significantly complemented performance of pro-SFTPb in both the discovery and validations sets, with a combined area under the curve in the validation set of 0.808 ( $P=0.001$  DAS + pro-SFTPb *vs.* pro-SFTPb alone) (Figure 1B).

While these risk marker studies show promise for identifying subjects with increased risk based on their biomarker profiles that otherwise may not be eligible for screening, much work remains to be done to determine an optimal, cost effective approach to assess risk based on a combination of biomarkers and subject characteristics.

## Conclusions

It is likely that biomarker panels will emerge in the near future that have relevance to lung cancer early detection

that will be useful in clinical practice. As experience is gained, it is also likely that improvement in biomarker testing will ensue through better biomarker combinations or through increased understanding of the population(s) that would best benefit from tailoring of biomarkers, for instance patients with COPD, light smokers ineligible for LDCT-based screening, or those with indeterminate nodules discovered incidentally or after LDCT-based screening. Biomarker panels will need careful validation among populations relevant to their intended application, which will be greatly facilitated by multi-center cooperation between lung cancer screening programs, ongoing clinical trials, and speciality diagnostic clinics.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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