A review of novel biological tools used in screening for the early detection of lung cancer

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ABSTRACT

Lung cancer is the most common cancer worldwide and causes more deaths per year than any other cancer. It has a very poor 5-year survival rate of 8–16%, partly because of comorbidity preventing curative treatments but mainly because of the disease presenting with symptoms only when it is at an advanced and incurable stage. When lung cancer is detected earlier and is amenable to radical treatments such as potentially curative surgery and radical radiotherapy, 5-year survival rates are much higher (up to 67%). Therefore reliable detection of lung cancer at this earlier (usually asymptomatic) stage of disease should be an important way to improve outcomes. This review discusses the principles of screening with respect to lung cancer, concentrating mainly on the biological modalities used to detect it. The lack of impact achieved by early studies using sputum cytology (in conjunction with chest radiographs) is described, and then newer technology used to measure other biomarkers in sputum, serum, exhaled breath and bronchial mucosa to diagnose (early) lung cancer is detailed. Many techniques show promise, but debate continues about which population to screen and what is the most (cost) effective modality to use. Moreover, no single biomarker or combination of biomarkers in screening has yet been shown to reduce lung cancer mortality in large prospective randomised studies.

Lung cancer is the most common cancer worldwide in terms of both incidence and mortality, with 1.3 million new cases being diagnosed each year and 1.2 million deaths, with Europe and North America having the highest incidence and prevalence rates.^{1,2} Compared with other common cancers such as prostate and breast cancer, lung cancer has much lower survival rates, and, despite advances in radiological tests, particularly CT and positron emission tomography scanning, surgical techniques and postoperative management, radiotherapy delivery and new chemotherapeutic agents, the long-term survival from lung cancer has remained remarkably constant and has not improved significantly over the last 20 years.³ Apart from lowering its incidence by reducing cigarette smoking, the key to improving lung cancer survival at present is to diagnose it at an earlier stage. This has led to decades of research to identify a suitable screening technique in order to reduce mortality.

The objectives of this article are to describe the principles of screening and show how they are relevant to lung cancer. We will briefly discuss purely radiological attempts at screening with low-dose spiral CT (LDCT) and how simple radiology (chest radiographs (CXR)) has been combined with sputum cytology, before describing in more

detail how novel biological techniques (or biomarkers) have been tried and are still being applied in the detection of lung cancer. We will also explain some of the biological reasoning for the use of certain biomarkers and, in particular, how they are based on a better molecular understanding of lung carcinogenesis.

PRINCIPLES OF SCREENING

Our improved understanding of the cellular and subcellular changes that lead to cancer, combined with advances in biological and computer technology, has led to a significant widening of screening techniques. When considering a disease entity for screening purposes, one has to consider the principles of screening. Screening is an important process in medicine, used to identify a disease within a defined population to improve health outcomes. In 1968, the World Health Organization published guidelines on the principles of screening.⁴ For a screening tool to be effective, the following principles must be adhered to.

- (A) **The condition should be an important health problem.** Lung cancer is the most common cancer worldwide with 1.3 million new cases diagnosed each year.²
- (B) **The disease should have significant mortality and morbidity.** Despite variations worldwide in the 5-year survival rate in lung cancer, collectively the figures show how devastating the disease is. The overall 5-year survival rate in America is 16%, although this figure is confounded by only including a quarter of the population and excludes those without histological confirmation of diagnosis.⁵ In Europe, lung cancer has an overall 5-year survival of only 10.9% (compared with a 5-year survival rate of 79% for breast cancer and prostate cancer of 78%).⁶
- (C) **There should be a latent phase of the disease.** Lung cancers usually present when a patient develops symptoms, so in most cases, the cancer is already advanced at the time symptoms develop. Screening aims to detect these cancers in the earlier asymptomatic/latent phase.
- (D) **Intervention earlier in the disease process should improve outcomes.** The 5-year survival rate for patients with stage IA non-small cell lung cancer (NSCLC) who have undergone complete surgical resection of the tumour is much higher at 67%.⁷
- (E) **The screening test itself should have certain characteristics.** It must be sensitive (to avoid false negatives and inappropriate reassurance) and specific (to avoid too many false positives leading to unnecessary worry

and further expensive/invasive testing). The test should also involve little risk of harm (or much less than the disease), and should be acceptable to the majority of the population to be screened.

- (F) **The cost of finding a case using the screening technique should be considered in relation to medical expenditure as a whole.**

LDCT AND LUNG CANCER SCREENING

There have been many observational studies assessing the feasibility of using LDCT in detecting lung cancer at an earlier stage and thus allowing curative treatment. The advantage of using such technology is that it allows detection of much smaller nodules (2–3 mm), which would almost certainly not be visible on CXR. As a result, a large number of early cancers have indeed been detected; a large study has found some survival advantage, but this was not a randomised controlled trial.⁸ Other studies with LDCT screening have reported no effect in reducing mortality.^{9–10} The likely explanation for this is a large number of indolent cancers, which would not have led directly to death, being detected. The National Lung Screening Trial (NLST), a randomised control study looking at LDCT screening techniques for lung cancer, will be reported in the next 2–5 years.¹¹ A detailed review of radiological screening is not the main focus of this article and is available elsewhere.^{12–15}

The rest of this article reviews several biological techniques based on sampling body tissues, which have been studied as potential screening tools for lung cancer. We describe commonly used sputum cytological techniques before concentrating on novel biological tools, which have been applied to sputum, serum, exhaled breath and bronchoscopy. Table 1 summarises these biological screening tools.

SPUTUM CYTOLOGY AND SCREENING IN LUNG CANCER

Sputum is a good biological specimen for screening because it is readily accessible and the technique is therefore non-invasive. Sputum cytology (ie, microscopic examination looking for abnormal cells) in combination with CXRs has been used as a screening tool in three large studies sponsored by the National Cancer Institute. The Memorial Sloan-Kettering Study, The John Hopkins Study and Mayo Lung Project have, in total, randomised over 30 000 male smokers (usually over 45 years), to various combinations of CXRs with or without sputum cytology screening, at baseline and repeated at interim periods over 3–5 years. Asymptomatic, often operable, carcinomas were diagnosed at baseline in 0.5–0.8% of the participants, and also further incident tumours during the study. Early results looked promising, but all three studies concluded that adding regular sputum cytological screening offered no advantage to CXRs alone, but most importantly there was no survival advantage to the screened groups compared with historical controls or the

unscreened population.^{16–23} A large study from Czechoslovakia involving 6300 heavy smokers also found no significant population survival advantage of performing regular CXRs (with or without regular sputum cytology) over 3 years, even after 15 years of follow-up.^{24–25} An interesting and consistent finding from the studies is the high frequency of detection of squamous cell carcinoma in the sputum cytology groups.

Together, these studies suggest that adding regular traditional sputum cytology (biological) screening to CXRs offers no further long-term survival advantage for the screened population. Some of the improved early survival rates were probably due to a combination of overdiagnosis, lead-time bias and length-time bias, therefore alternative screening approaches need to be considered. Since these early studies, improvements have been made in CXR technology, and, as a result, a large randomised control study reassessing the role of annual CXRs is in progress. The Prostate, Lung, Colorectal and Ovarian Cancer screening trial (PLCO study) has been instituted by the National Cancer Institute and includes over 150 000 men and women aged 55–74, who were allocated to two trial arms. One arm is intervention (screening) and the other is the control arm, with normal healthcare routines continued. The long-term survival outcomes from this study are pending.²⁶

THE PRINCIPLE OF BIOMARKERS AND LUNG CANCER: UNDERLYING HISTOLOGICAL AND CELLULAR CHANGES

The understanding of the molecular biology of lung cancer is rapidly developing, and new techniques are based on understanding the key cellular changes that occur in lung cancer. Moreover, non-cytological/non-radiological biomarkers are being evaluated in monitoring and predicting treatment response because of our understanding of these cellular processes.

Both squamous dysplasia and carcinoma in situ are pre-neoplastic abnormalities, which often (but not always) lead to squamous cell carcinoma.²⁷ Preneoplastic abnormalities in adenocarcinoma and carcinoid are different and include atypical adenomatous hyperplasia and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia.²⁷ When developing biomarkers, researchers have found changes in gene expression and chromosome structure within these preneoplastic lesions. These include mutations in the p53 and *ras* genes that are associated with hyperproliferation and loss of cell cycle control, aberrant gene promoter methylation, increased vascular growth and altered protein expression.^{27–29}

Unfortunately, the variability of these changes and our inability to reliably detect them has meant that no single specific biomarker has yet been identified with adequate sensitivity, specificity and reproducibility. Various groups are now developing high-throughput techniques, to apply panels of biomarkers associated with these cellular changes for detecting and monitoring lung cancer.

SPUTUM BIOMARKERS AND SCREENING FOR LUNG CANCER

Gene promoter methylation

This is a very promising modality for lung cancer screening. Early changes in the development of lung cancer include methylation of promoter sequences in different tumour suppressor genes. These are associated with the silencing of transcription and inactivation of these tumour suppressor genes, playing a crucial role in triggering malignant transformation and progression.²⁹ Methylation is detected by specific PCR analysis. Hyper-methylation was investigated in a small “proof-of-concept”

Table 1 Summary of biological techniques reviewed

Tissue sample/procedure	Screening tool
Sputum	Cytology
	Gene promoter methylation
Serum	Circulating DNA
	Vascular endothelial growth factor
	Proteomics
Breath	Volatile organic compounds
Bronchoscopy	Autofluorescent bronchoscopy
	Genetic testing of epithelial cells

study looking at methylation of p16 and *O*⁶-methylguanine–DNA methyltransferase (MGMT) gene promoters in a high-risk population. Twenty-one patients with squamous cell carcinoma were investigated. The presence of one or both gene promoters was found in all 21 samples irrespective of whether they were taken up to 3 years before or at the time of diagnosis. This compares with 15% (methylation of p16) and 25% (methylation of MGMT) in 123 controls, deemed high risk because of a smoking history and/or radon exposure. Interestingly, 48% of the patients with squamous cell cancer had methylation of both genes compared with only 4% of controls ($p < 0.001$). On follow-up, three lung cancers were diagnosed in the controls between 1 and 3 years after sputum collection, with the MGMT gene being methylated in two of the subjects.³⁰

Belinsky *et al*³¹ postulated that a panel of methylated genes would yield greater sensitivity and specificity. They recruited subjects older than 25 years, with ≥ 30 pack-year smoking history and airways obstruction with a forced expiratory volume in 1 s (FEV₁) $\leq 75\%$ and FEV₁/FVC (forced vital capacity) ≤ 0.75 . They excluded subjects with any cancer diagnosed in the previous 5 years. A total of 3259 subjects provided pooled sputum for 3 days in one container, and the next 3 days in a second container. The sputum from the second container was used as samples for the study. There were 1353 cohort deaths, and 182 subjects were diagnosed as having lung cancer. Once sufficient quality of DNA was analysed, the study cohort consisted of 98 subjects (cases), and the non-cancer cohort was used to form a group of 92 controls matched for age, gender and month of enrolment. Although there were 26 current smokers in the control group compared with 42 in the cases, the total number of smoking pack-years was similar. Fourteen genes were analysed for promoter methylation, including p16 and MGMT. It was found that six of the 14 genes (p16, MGMT, DAPK, RASSF1A, PAX5 β and GATA5) were individually associated with a $>50\%$ increased risk of lung cancer. Sputum collected within 18 months of a diagnosis of lung cancer had more methylated gene promoters than sputum from the same subject collected more than 18 months before the diagnosis. The concomitant methylation of three or more genes was associated with a 6.5-fold increased risk of lung cancer, with receiver operating characteristics of a specificity of 64% and sensitivity of 64%.

This is the first study to show how a panel of genes can be used as potential biomarkers in screening a high-risk population for lung cancer and suggests an accumulative “hit” effect. The researchers concluded that their level of specificity is not yet high enough for prospective screening studies, but called for more evaluation of candidate gene panels, especially as technology improves.

SERUM BIOMARKERS AND SCREENING FOR LUNG CANCER

Serum biomarkers have been used to screen for and monitor different cancers. These include prostate-specific antigen for prostate cancer, CEA and CA19-9 in colonic cancer and CA125 in ovarian cancer. More recently, serum has been analysed as a screening biofluid for lung cancer.

Circulating DNA and genetic changes

Raised concentrations of circulating cell-free DNA in patients with cancer were first reported in 1987. Although the precise mechanism of DNA release into the blood remains obscure, it appears that much of this circulating DNA is derived from apoptotic and necrotic tumour cells.³² Patients with lung cancer

show genetic and epigenetic changes consisting of chromosome loss, oncogene activation and tumour suppressor gene methylation,^{33–34} so the concentrations of this circulating DNA may allow development of specific markers. Concentrations of circulating cell-free DNA are generally higher in patients with cancer than in healthy controls.^{35–36}

k-ras and p53 are well-characterised common mutations in human lung cancer and preneoplasia. *k-ras* mutations have been found in the circulating DNA of 20–30% of patients with lung cancer, with only one study detecting the mutation in the control group, although the characteristics of the controls were not specified.^{37–40} p53 mutations have been found in 10–30% of patients with lung cancer compared with minimal detection in the healthy control groups.⁴¹ One study found p53 mutations in 41% of lung tumours, with the identical mutation identified in the plasma of 73% of them.⁴²

Vascular endothelial growth factor (VEGF)

Recent preliminary data showed significantly ($p = 0.033$) increased VEGF concentrations (measured using enzyme immunoanalysis) in seven patients with NSCLC compared with five healthy smokers and seven controls.⁴³

Proteomics

Proteomics is the study of proteins, and two approaches have been applied to lung cancer. The first, protein profiling, is where patterns of protein expression are used, and the alternative is identifying individual proteins. Yang *et al*⁴⁴ analysed serum from 158 patients with lung cancer and 50 controls using a mass spectrometry technique. Seventy-four lung cancer serum samples and 20 healthy controls were used to develop a training set. From this, a specific pattern consisting of five protein peaks was chosen as biomarkers that could diagnose NSCLC, and this pattern was used, in a blinded fashion, in an attempt to discriminate the remaining serum samples of 84 patients with lung cancer and 30 healthy controls. The pattern of proteins yielded a sensitivity of 87% and specificity of 80%. These techniques are also being evaluated to assess for premalignant changes in high-risk people.⁴⁵

BREATH BIOMARKERS AND SCREENING FOR LUNG CANCER

Volatile organic compounds (VOCs) in breath

Exhaled breath samples are even more accessible than sputum. More than 200 different measurable chemicals are exhaled in the human breath.⁴⁶ Gordon *et al*⁴⁷ studied VOCs in exhaled air from 12 patients with histologically confirmed NSCLC and nine healthy controls (two were heavy smokers). Gas chromatography combined with mass spectrometry identified four peaks representing individual VOCs in more than half of the patients with lung cancer, which were completely absent in the control group. Gordon *et al* suggested that unique VOCs are exhaled in the breath of patients with lung cancer, which may have potential diagnostic importance, and recommended that this technique be further tested in a larger population.

Phillips *et al*⁴⁸ collected exhaled breath in 108 fasting patients about to undergo a bronchoscopy for an abnormal CXR. Lung cancer was confirmed histologically in 60 patients (50 NSCLC, 10 small cell lung cancer) and excluded in 48. Many VOCs were common to both sets of breath samples, but a group of 22 of these VOCs could be used to distinguish between lung cancer and control cases according to risk weighting attached to the test. For example, the VOCs had 100% sensitivity (ie, no false negatives) and 81% specificity for stage I lung cancer if the

receiver operator accepted a post-test probability of 0.46. A post-test probability of 0.9 yielded lower (67%) sensitivity but 100% specificity (no false positives). The abnormal VOC peaks consisted of mainly alkanes and benzene derivatives. Smoking status was self-reported and did not account for the benzene derivatives, as these were also present in the breath of non-smokers and ex-smokers. There were no significant differences in the sensitivity and specificity of VOCs between early and advanced lung cancer. Phillips *et al* also concluded that patterns of VOCs could act as a “fingerprint” for lung cancer, but called for validation in a larger general population.

The same researchers compared VOCs in patients with biopsy-proven primary lung cancer ($n = 67$), non-lung cancer metastasising to the lungs ($n = 15$) or abnormal CXRs but no histological evidence of lung cancer ($n = 5$), and healthy volunteers from the general population ($n = 41$). The breath test identified over 80 different alkanes and monomethylated alkanes used to generate a predictive model using a panel of nine VOCs. These nine VOCs, in combination, yielded a sensitivity of 90% and a specificity of 83% (using a post-test probability of 0.5) of identifying primary or secondary lung cancer. There were only minor differences when subjects were stratified according to smoking history, histological cancer type or staging.⁴⁹ The authors believed that the test was sensitive, cost-effective and easy to perform and could be used to complement other modalities, but again called for further evaluation.

Italian researchers measured 13 VOCs (seven aliphatic and six aromatic compounds) in 36 NSCLC cases, 25 controls with stable mild-moderate chronic obstructive pulmonary disease (COPD), 35 asymptomatic smokers and 50 healthy non-smokers with normal spirometry.⁵⁰ Although no single VOC could distinguish NSCLC from the other groups, the panel of VOCs performed with an overall sensitivity of 72% and specificity 94%. Twenty-six of the patients with NSCLC agreed to have repeat VOC analysis after surgical resection, which revealed a significant reduction in their exhaled isoprene and decane concentrations. Poli *et al*⁵⁰ still concluded that the use of VOCs alone could not be recommended at this stage in the early detection of lung cancer.

The studies discussed thus far involve mass spectrometry of the VOCs. New techniques using VOCs have recently been developed. One such system consists of a colorimetric sensor array, which has 36 spots made up of different chemically sensitive compounds. The colour of the spot changes depending on the chemical it comes into contact with. A study to assess this principle was performed by Mazzone *et al*.⁵¹ The study included 143 subjects: 49 had NSCLC, 73 had various chronic lung diseases such as COPD, idiopathic pulmonary fibrosis, pulmonary arterial hypertension and sarcoidosis, and there were 21 controls. Once a prediction model had been developed using 70% of the subjects, it could be used to predict the presence of lung cancer in the remaining 30% with a sensitivity of 73.3% and specificity of 72.4%. Although this suggests only moderate accuracy of the diagnosis, the study does prove the potential of this technique. Mazzone⁵² has recently published a review of VOCs in lung cancer.

BRONCHOSCOPY AND SCREENING FOR LUNG CANCER

Many lung cancers are diagnosed by histological analysis of endobronchial samples obtained by fibre-optic bronchoscopy. The role of bronchoscopy has expanded over the last few years and now encompasses localised treatments such as laser therapy, stent insertion and brachytherapy. As well as obtaining

Key learning points

- ▶ Lung cancer is the most common cancer worldwide.
- ▶ Five-year survival rates are significantly worse than for breast and prostate cancer.
- ▶ Screening using standard techniques such as sputum cytology and plain chest radiographs have not been shown to improve mortality.
- ▶ Screening studies using CT are ongoing.
- ▶ Newer techniques measuring biomarkers in easily accessible tissues such as serum, sputum and exhaled breath, and even in less easily obtained tissue such as endobronchial samples, show some promise in detecting (early) lung cancer.
- ▶ Overall, no single cheap and reliable test has yet been identified for effective screening of lung cancer in large population studies, but work is in progress.

a histological diagnosis and allowing some treatment, bronchoscopy may have a role in screening for lung cancer.

Autofluorescent bronchoscopy

The principle behind autofluorescent bronchoscopy is that, when blue light (wavelength 380–460 nm) is shone on to abnormal mucosa, fluorescence is reduced (compared with normal mucosa), allowing more lesions to be identified (particularly premalignant ones) compared with the use of shining standard white light. A review suggested that autofluorescent bronchoscopy (in experienced hands) can diagnose carcinoma in situ in 1.6% of cases deemed high risk, and moderate and severe dysplasia in 19% of current heavy or former smokers with sputum atypia.⁵³ Moreover, the preinvasive lesions were small, with 55% being ≤ 1.5 mm in greatest diameter. Over 1000 cases comparing white light against autofluorescent bronchoscopy found 40% of preinvasive lesions detected by white light bronchoscopy alone, but ~80% of lesions were detected by adding autofluorescent bronchoscopy.⁵⁴

Loewen *et al*⁵⁵ combined autofluorescent bronchoscopy, sputum cytology and spiral CT for surveillance in a very high-risk group. Two or more of the following criteria had to be fulfilled: ≥ 20 pack year smoking history; asbestos-related lung disease on CXR; $FEV_1 < 70\%$ of predicted, treated aero-digestive cancer with no evidence of disease for > 2 years. A total of 186 patients were enrolled, and 169 completed the baseline tests; 66% of the patients had squamous metaplasia or worse and 7% had a diagnosis of cancer at baseline. Sputum cytology missed 100% of the dysplasia and 68% of the metaplasia detected by autofluorescent bronchoscopy, and failed to detect any cases of carcinoma or carcinoma in situ. Patients who had pulmonary nodules on spiral CT scan were 3.2 times more likely to exhibit premalignant changes on autofluorescent bronchoscopy ($p < 0.001$). The study concluded that autofluorescent bronchoscopy should be carried out on high-risk people regardless of the results of conventional sputum cytology.

Other studies have found no increased detection rates of carcinoma in situ or dysplasia using autofluorescent bronchoscopy compared with white light bronchoscopy in current or former smokers,⁵⁶ suggesting that autofluorescent bronchoscopy is best used in highly selected cohorts with sputum atypia or when multiple risk factors for lung cancer are present.

Current research questions

- To identify a biomarker that is cost-effective and with good enough accuracy to be used as a screening and monitoring tool to impact on lung cancer survival.
- To consider combinations of biomarkers, possibly using more than one tissue or combined with low-dose CT scans in screening for lung cancer.

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Genetic testing of epithelial cells from endobronchial brushings

Spira *et al*⁵⁷ investigated whether gene expression profiling of large airway epithelial cells could be used as a biomarker in histologically normal bronchial mucosa obtained by bronchial brushing. A total of 129 current or former smokers who underwent fibre-optic bronchoscopy for a clinical suspicion of lung cancer were followed-up until a final diagnosis was made. Sixty were diagnosed as having lung cancer (bronchoscopy or subsequent lung biopsy yielded lung tumour cells), and 69 without lung cancer (investigations yielded a non-cancer pathology or the radiological abnormality resolved). From a training set ($n = 77$), an 80-gene biomarker panel was identified and then tested prospectively on the remaining 52 cases, obtaining an accuracy of 83% (80% sensitivity, 84% specificity). Among the non-diagnostic bronchoscopies ($n = 92$), the accuracy of the panel of gene biomarkers was 85% (89% sensitive, 83% specific). By adding the biomarker panel to the traditional tests, the diagnostic sensitivity was improved to an impressive 95% with a 95% negative predictive value. Spira *et al* also concluded that gene expression in cytologically normal bronchial epithelial cells can serve as a lung cancer biomarker.

CONCLUSIONS

Lung cancer is common, and only a small proportion of patients survive more than 5 years. Screening studies using serial CXRs and sputum cytology examination (and combinations of them) have not improved group mortality. Screening with LDCT scanning is still under review. Importantly, a better understanding of the biology of lung carcinogenesis and improved techniques in DNA and chemical analyses are being used to develop cost-effective, high-throughput technologies on samples of readily accessible tissues such as blood, sputum and even exhaled breath. As yet, none have been proven in large studies, but, as these novel biological techniques continue to evolve, it is hoped that, in the future, combinations of biomarkers will lead

to non-invasive, cost-effective screening tools to improve outcomes in this common and devastating disease.

TRUE/FALSE QUESTIONS (ANSWERS AFTER THE REFERENCES)

1. Lung cancer is the most common cancer worldwide.
2. For a screening modality to be effective, it must have a high sensitivity and specificity.
3. Standard cytology and plain chest radiograph screening studies have shown an increased survival advantage in the screened group compared with the control “non-screened” group.
4. Patients with lung cancer may have a different profile of volatile organic compounds in their breath from those without lung cancer.
5. The addition of autofluorescent bronchoscopy to traditional white light bronchoscopy may double the chances of diagnosis of a preinvasive lesion.

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Answers

(1) T; (2) T; (3) F; (4) T; (5) T

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