SERIES "HIGHLIGHTS IN LUNG CANCER"
Edited by C. Brambilla and S. Spiro
Number 5 in this Series

Role of biomarkers for early detection of lung cancer and chemoprevention

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ABSTRACT: Lung cancer is the leading cause of cancer deaths in developed countries. The poor prognosis associated with this disease is closely related to the fact that most lung cancer patients are not identified until their malignancy has reached an advanced stage.

Recent advances have added to the understanding of the morphological and molecular characteristics of preinvasive bronchial lesions and early lung cancers. Such information is being used to provide new tests for the detection of lung cancer at early or preinvasive stages, and for identifying targets for therapeutic intervention that can prevent progression to advanced disease. Laser induced fluorescence endoscope bronchoscopy has improved the sensitivity with which preinvasive dysplastic bronchial lesions and early invasive malignancies can be detected. Morphological features of such lesions have been described and can be monitored by follow-up bronchoscopies in order to validate potential chemoprevention treatments.

Distinct morphological characteristics such as angiogenic squamous dysplasia also suggest that processes like angiogenesis are present early in the development of lung cancer. Furthermore, tissue obtained from these early lesions has been used to describe alterations in the expression of a number of factors that distinguish these early lesions from normal bronchial epithelium. This could provide molecular markers and targets for the detection and treatment of early lung cancer. Studies to detect these alterations by polymerase chain reaction and/or immunhistochemical analyses of easily obtained specimens such as sputa are helping identifing molecular markers that could be utilized in effective screening programmes.

The current article reviews new findings regarding the molecular biology of preinvasive bronchial lesions and early lung cancers, and describes new developments regarding their application in the early detection and chemoprevention of lung cancer.
Eur Respir J 2002; 19: 1151–1158.

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Lung cancer is the leading cause of cancer deaths in the USA in both males and females [1]. The high mortality associated with this disease is primarily due to the fact that the majority of lung cancers are not detected until they have progressed to an advanced stage. More than two-thirds of lung cancers are associated with lymph-node metastases at the time of diagnosis [2]. The 5-yr survival of patients with clinical stage II–IV disease is poor, ranging from 40% down to 5% [3]. In order to reduce the mortality associated with lung cancer, new techniques must be developed to diagnose and treat early invasive or preinvasive disease. The development of such tools could lead to the establishment of routine screening protocols in well-defined high-risk patient populations. Chest radiography and sputum cytology have

been the primary tools used for establishing diagnoses of lung cancer. In the 1970s several large clinical trials were performed to evaluate the efficacy of using these two methodologies together in lung cancer screening programmes [4–6]. Although both techniques have been demonstrated to identify malignancies well before lung cancer becomes clinically symptomatic [7, 8], the implementation of screening programmes using these testing modalities failed to show a reduction in lung cancer mortality in large clinical trials. To date, no lung cancer screening programme has been approved for general clinical application. While many have raised questions regarding the design and interpretation of these original clinical trials, the need to develop new techniques for early diagnosis and effective therapy is widely recognized.

Highly sensitive and specific tests for the early diagnosis of lung cancer and its precursor lesions are needed in order to establish effective lung cancer screening programmes. Furthermore, it is important to be able to perform these tests on easily obtained specimens in a cost-effective manner. Finally, effective new therapies will need to be developed to treat patients identified in the screening programmes. Recent work has begun to define the molecular characteristics of preneoplastic and early invasive bronchial lesions. In addition to providing an understanding of the biology of these early lesions, the information gathered through these efforts should allow for the development of new diagnostic and therapeutic strategies that will ultimately provide the tools needed to reduce the devastating toll associated with lung cancer. In this article new advances in the understanding of the biology of early lung cancer associated lesions will be reviewed and some of the promising new data regarding diagnosis and treatment of these lesions will be discussed.

Biology of premalignant and early invasive bronchial lesions

In recent years numerous studies have provided a wealth of information regarding the histological and molecular characteristics of premalignant changes in bronchial mucosa [9]. These various epithelial lesions are thought to represent a histological spectrum which is now included in the World Health Organization (WHO) classification of lung tumours (fig. 1). The earliest lesions include reserve cell hyperplasia and squamous metaplasia without associated cellular atypia/dysplasia. Because these lesions spontaneously regress with smoking cessation, they are believed to represent reactive changes rather than true premalignant lesions [10, 11]. This view is supported by findings that show reserve cell hyperplasia and squamous metaplasia are not consistently associated with molecular alterations which distinguish them from normal bronchial epithelium and are frequently found in association with chronic inflammation. Conversely, low and high grade dysplasia and carcinoma *in situ* (CIS) are believed to be true premalignant lesions associated with increased risk for the development of lung carcinoma. While the degree of increased risk associated with low-grade dysplasias is unclear, the presence of high-grade dysplasia and CIS as identified in sputa or bronchial tissue have been demonstrated to be associated with a significant increased risk of subsequent development of lung cancer [12, 13]. In one study it was demonstrated that, depending on the degree of dysplasia (moderate *versus* severe), 10–40% of patients diagnosed with high-grade dysplasia in sputa specimens were observed to subsequently develop cancer in a period of up to 9 yrs following the diagnosis of dysplasia [12]. A study conducted by SACCOMANO *et al.* [7] involving multiple specimens
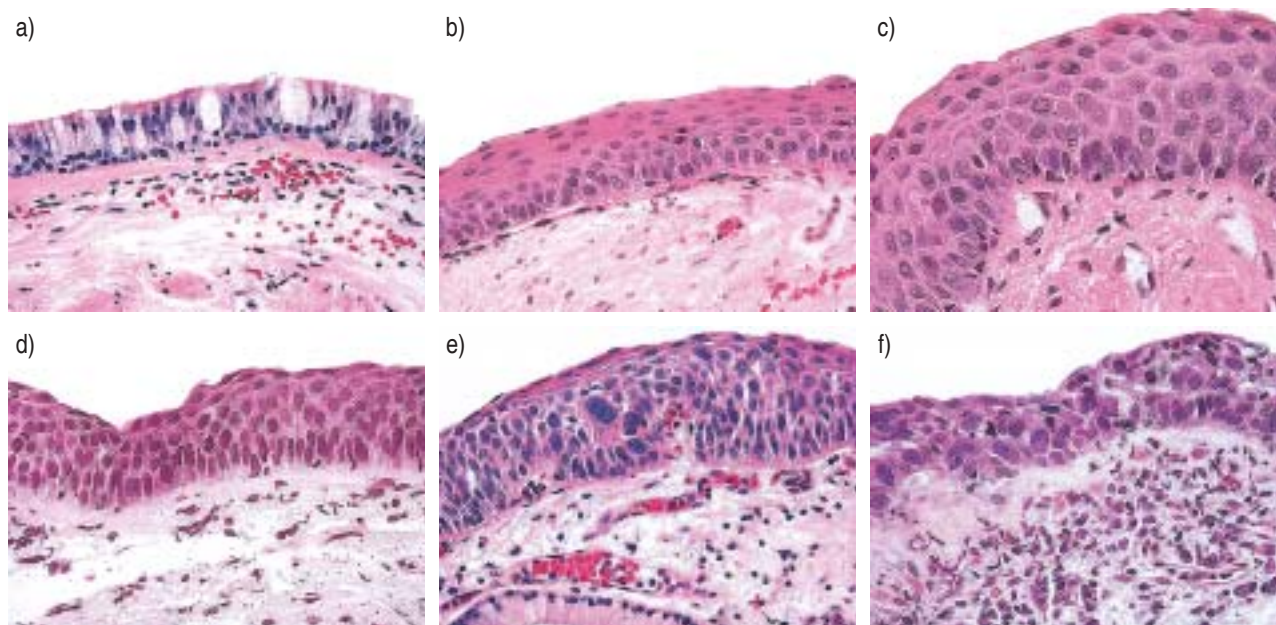

Fig. 1. – Grading of morphological changes in bronchial epithelium used for classification scheme applied in chemoprevention and other studies at the University of Colorado Cancer Centre. a) Normal (grade 1), b) squamous metaplasia (grade 2), c) mild dysplasia (grade 4), d) moderate dysplasia (grade 5), e) severe dysplasia (grade 6) and f) carcinoma *in situ* (grade 7).

from 6,000 male patients demonstrated that cytologic atypia could be identified in sputum specimens an average of 4–5 yrs before the development of frank lung cancer. Another study showed that the majority of a small group of patients with biopsy-proven CIS subsequently developed invasive cancer [13]. In addition, the observation that high-grade dysplasia and CIS seldom regress even after smoking cessation further demonstrates the serious potential associated with these lesions [14].

Additional observations regarding histological characteristics and molecular alterations associated with these premalignant bronchial lesions also support their central role in the development of lung cancer. Angiogenic squamous dysplasia (ASD) is a term that has been introduced to describe a frequent histological feature seen in bronchial dysplasias [15, 16]. ASDs demonstrate papillary vascular structures that protrude into overlying dysplastic bronchial epithelium, and these lesions have been demonstrated to be associated with an increase in the number of subepithelial microvascular structures as compared to normal bronchial epithelium [15]. Furthermore, immunohistochemical studies have shown expression of the angiogenic mediator vascular endothelial growth factor in dysplastic bronchial epithelium [17, 18]. Angiogenic activity is required for the growth of malignant tumours. Thus the demonstration of this activity in premalignant lesions of the airways further supports the malignant potential attributable to bronchial preneoplasia and could have significance in relation to the treatment of these lesions. Dysplastic lesions are found in the airways of most patients with lung cancer in both contiguous and remote sites in relation to the invasive cancer. Molecular studies comparing dysplasias and invasive cancers from the same resection specimen have also revealed evidence that supports the role of dysplasia as a precursor lesion in the development of invasive cancer. Many of the dysplastic lesions in these specimens display loss of heterozygosity (LOH) in chromosomal sites that are identical to those found in the associated invasive cancer [19]. Similar mutations in the tumour suppressor gene, p53, can also be found in lung cancers and their corresponding dypslasias, however these concordant findings are much less frequent than those found in LOH analyses suggesting that p53 mutations may represent a later change in the progression to invasive cancer [19]. Such observations have also provided information pertinent to the understanding of the mechanisms by which lung cancer develops.

The development of invasive lung cancer is believed to involve two distinct but complimentary processes. Exposure of the lower airways to carcinogens in tobacco smoke leads to widespread cellular and molecular changes over broad mucosal expanses, a process first described in head and neck cancer [20] and referred to as "field cancerization". This process is associated with the stepwise accumulation of mutations and epigenetic alterations that lead to dysplasia and eventually to invasive carcinoma, a process referred to as "multistep carcinogenesis". Exposure of airway epithelium to repeated high doses of carcinogens in tobacco smoke may lead to the establishment of multiple lesions, each derived from a cell that has acquired unique mutations in comparison to other sites. This concept is supported by findings in a study of multiple primary lung tumours that demonstrated differences in the genetic alterations associated with each tumour [21]. Alternatively, it has been suggested that multiple airway epithelial lesions could be established from a single progenitor cell that has undergone mutation or epigenetic change and subsequent clonal expansion. In this model, cells derived from the original clone of altered cells establish new premalignant lesions at sites remote from the original site. The observation of identical p53 mutations in respiratory epithelium at widely dispersed sites within the airways of a lung cancer patient supports this concept [22]. These two hypotheses are not mutually exclusive and probably occur in combination. Regardless of the mechanism by which the premalignant lesions are established, the development of lung cancer would then follow *via* multistep carcinogenesis at the site in which the necessary type and number of additional genetic/molecular alterations occur.

Bronchial dysplasias are believed to be direct precursors of squamous cell carcinoma of the lung, however the role they might play, if any, in the development of other nonsmall cell and small cell lung carcinomas is unclear. In the case of adenocarcinoma, a separate lesion referred to as atypical adenomatous hyperplasia (AAH) has been implicated as a precursor of these invasive glandular cancers. Studies have shown that AAH frequently demonstrates LOH, aneuploidy, K-ras mutations and less frequent alterations of p53 expression similar to bronchial dysplasias [23, 24]. Small peribronchiolar lesions demonstrating neuroendocrine features and dubbed neuroendocrine hyperplasias have been suspected to be precursor lesions of small cell carcinoma but have not been definitively associated with the subsequent development of small cell carcinoma at this point [25]. Finally, a lesion described as "respiratory bronchiolitis" many years ago by AUERBACH, *et al.* [10] was found with sufficient frequency to suggest a possible, but largely unexplored, role in lung carcinogenesis .

Biomarkers of premalignant and early lung cancer lesions

A number of molecular alterations have recently been identified in premalignant bronchial lesions and early lung cancer (table 1). Altered expression of oncogenes, tumour suppressor genes (TSG) and deoxyribonucleic acid (DNA) repair genes frequently found in established lung cancer are now being found in varying degrees in dysplastic and minimally invasive lung lesions also. In addition, structural alterations (*i.e.* deletions, promoter methylation, *etc.*) to these genes that distinguish malignant tissue from normal bronchial epithelium are frequently present in these early lesions. Full characterization of these alterations in early lung lesions is beginning to provide promising information regarding both the identification of biomarkers that could be used in lung cancer screening programmes and molecular pathways that

Table 1. – Molecular alterations in various stages of bronchial carcinogenesis

Molecular alteration	Frequency %
Early changes[#]	
3p LOH/small telomeric deletions	80
Microsatellite alterations	50
9p21 LOH	70
Telomerase dysregulation	80
MYC overexpression	60
Intermediate changes[¶]	
8p21–23 LOH	80
Neoangiogenesis	40
Loss of FHIT immunostaining	40
p53 LOH	70
Aneuploidy	80
Methylation	100
Late changes[+]	
TP53 Mutations	70
5q21 APC-MCC LOH	30
K-ras mutation	20

LOH: loss of heterozygosity; FHIT: fragile histidine triad. [#]: found in normal hyperplastic and metaplastic epithelium in smokers; [¶]: generally first detected in bronchial dysplasias; [+]: usually only found in carcinoma *in situ* and invasive carcinoma.

could be targeted in therpeutic interventions aimed at preventing the development or progression of lung cancer.

TSGs have been some of the first and most thoroughly studied genes as potential early markers of lung cancer. Mutations of the p53 gene are found in many lung cancers, and studies have shown that these mutations can be detected in some of the sputa samples from patients with p53 mutation positive cancers [26, 27]. However, since p53 mutations are absent in significant numbers of lung cancers, and these mutations can be detected in less than one-half of sputa samples from patients with these p53 mutation positive cancers, it appears that screening using these analyses would not be sufficiently sensitive. Interestingly, in comparison to the rate of detection of p53 mutations, immunohistochemical studies have shown a higher prevalence of p53 overexpression in biopsies of premalignant bronchial lesions [28]. A study by BRAMBILLA *et al.* [29] has suggested that premalignant lesions associated with p53 overexpression are more likely to progress to invasive cancer than those that are not associated with overexpression. However, whether sensitive assays of p53 overexpression could be applied to screening specimens such as sputa remains to be determined. A broader genomic based assay of TSG alterations has been employed in LOH analyses of lung cancers and precursor lesions. These assays identify allelic loss of known and putative TSGs that are thought to contribute to the development of lung cancers when inactivation of the remaining allele occurs through processes such as mutation or hypermethylation in clones of cells in which there has been an initial LOH. Recent work by WISTUBA *et al.* [30] has detected 22 LOH hotspots in small cell lung carcinomas and nonsmall cell lung carciomas, many of which may

be mutated in one-half or more of lung cancers. Furthermore, LOH at many of these sites can also be demonstrated in premalignant lesions [31–35]. However, because of the multiplicity of loci that are affected by LOH and the detection of LOH at very early stages of carcinogenesis, it is unlikely that LOH analysis will provide a practical screening tool. In addition to TSGs, overexpression of oncogenes, cell-cycle regulators and cell-proliferation markers such as c-myc and k-ras, cyclins D1 and E and MCM2 respectively have been described in both tumours and premalignant bronchial lesions [36–41]. Alterations in microsatellite markers have similarly been found in preinvasive and invasive lung lesions [42, 43]. Taken alone, none of these markers show very high sensitivity with overall detection in tissue reaching only 60–70% at best. However, if used in combination an acceptable level of sensitivity in analyses of screening specimens such as sputa may be achievable.

Immunohistochemical analysis of sputum samples is a technique that is being investigated as a screening tool for potential application regarding the expression of early markers of lung cancer. One protein that has been studied extensively using this methodology is heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1). Overexpression of this protein appears to be ubiquitous in lung tissue with squamous cell carcinoma and is also found in the majority of adenocarcinomas and other types of nonsmall cell carcinomas [44–46]. Studies applying this technique to sputa from patients with subsequently identified invasive lung cancer have demonstrated a 2–3-fold increase in sensitivity in detecting lung cancer as compared to standard sputum cytology or radiographical analysis [45, 47]. These hnRNP A2/B1 positive cells could be detected in the sputum on average 2 yrs before detectable radiographical and cytological changes were discovered in the patients. In addition, recent studies have shown that hnRNP B1 positive staining can be demonstrated in nearly two-thirds of bronchial biopsies showing histological changes of bronchial dysplasia [46]. Whether the hnRNP B1 positive dysplasias are associated with higher potential for progression to invasive carcinoma remains to be determined.

Polymerase chain reaction (PCR) analyses of sputa and other types of specimens that could be used in screening assays is another technique that shows great promise for application in the early diagnosis of lung cancer. This technique has been employed in the study of DNA promoter methylation status in lung cancer. Inactivation of genes implicated in the development of cancer by promoter hypermethylation is now recognized as a frequent event. A number of recent studies have identified numerous genes whose expression is altered in lung cancer due to methylation [48–51]. The growing list of genes whose expression in lung cancer appear to be affected by hypermethylation includes genes with a wide variety of functions such as p16, p15, retinoic acid receptor-β (RAR-β), RASSF1, fragile histidine triad (FHIT), tissue inhibitor of metalloproteinase 3, O6-methylguanine-DNA-methyltransferase (MGMT), death-associated protein kinase,

E-cadherin, p14 and glutathione S-transferase P1. Re-expression of some of these genes has been shown to inhibit *in vitro* colony formation and *in vivo* tumour formation in animal studies [51]. In addition, hypermethylation of p16 has been detected in premalignant bronchial lesions, and this alteration is found more frequently in lesions demonstrating higher degrees of atypia [48]. The prevalence of hypermethylation of these genes in lung cancer varies with the most frequently affected genes being altered in just over one-half of lung cancers. Thus, taken alone, assays of hypermethylation of any of these genes would not demonstrate a high enough degree of sensitivity to be useful in lung cancer screening. However, recently published work by PALMISANO *et al.* [52] has demonstrated that by using a more sensitive assay for promoter hypermethylation to concomitantly assay p16 and MGMT genes, 100% of sputa specimens taken from patients with squamous cell carcinoma can be demonstrated to display aberrant methylation. Furthermore, these changes could be detected in sputa collected up to 3 yrs before clinical diagnosis was established. Also of interest is the detection of p16 and MGMT gene hypermethylation in the serum of 75% of patients with the same changes in tumour tissue [53]. However, serum detection of p16 hypermethylation was found in only one of 10 patients with nonsmall cell lung cancer studied in a recent analysis [54]. Finally, treatment of cell lines demonstrating hypermethylation of the RAR-β gene with demethylation agents has been shown to re-establish RAR-β expression suggesting potential therapeutic applications [55].

Specimens for clinical testing

Most of the data discussed thus far regarding the early detection of lung cancer has concentrated on analysis of sputum specimens. However, other types of specimens have also been evaluated for their potential value in screening programmes. Serum samples have been utilized as sources for clinical testing in numerous disease processes and, due to the ease and low cost of collection, have also been studied extensively for their usefulness in establishing cancer diagnoses. Antibody, antigen and PCR based assays have been used to try to screen peripheral blood/serum in patients with lung cancer. Studies aimed at identifying epithelial cell-related antigens in serum have been shown to lack adequate specificity [56, 57]. Recently, a study that attempted to identify autoantibodies to c-myc and circulating c-myc antigens in the serum of lung cancer patients showed a low level of sensitivity [58]. More promising results have been published regarding the detection of tumour specific DNA alterations in serum. Minute, but elevated levels of circulating DNA have been detected in cancer patients as compared to normal individuals [59–61]. Using PCR based assays microsatellite instability has been detected in the serum of 93% of patients with similar alterations in their small cell carcinomas [62]. As mentioned earlier, promising but unconfirmed findings regarding hypermethylation

have also been reported in studies of serum from lung cancer patients [53].

Bronchoscopically obtained specimens have shown good potential as sources for molecular detection of lung cancer, although their value in screening analysis is tempered by the degree of invasiveness and cost associated with the procedure. Bronchoalveolar lavage (BAL) specimens obtained by re-aspirating saline infused into the airways of patients undergoing bronchoscopy should provide a specimen with a deeper epithelial component than found in sputum samples. Indeed, a recent study of BAL fluids revealed the detection of numerous tumour associated molecular alterations including frequent mutations in p53 and k-ras genes, and alterations in microsatellite loci and p16 promoter methylation status. Unfortunately, the sensitivity associated with the finding of these alterations was not as great as would ultimately be desired [63]. Similarly an analysis of p16 hypermethylation in BALs from a small group of patients with hypermethylated p16 gene in resected tumour tissue found a poor degree of sensitivity for detection in the corresponding BAL specimens [54]. Conversely, FIELDING *et al.* [64] demonstrated a high degree of sensitivity for detection of hnRNP A2/B1 overexpression in BAL specimens in patients with premalignant and malignant lung lesions. However, studies of BAL specimens have not demonstrated significant differences in comparisons to assays of sputa thus far. Bronchoscopically obtained biopsies have demonstrated a significantly increased sensitivity in detecting premalignant and early invasive lung lesions since the introduction of laser-induced fluorescence endoscopes (LIFE) [65]. Through this procedure dysplasias and early invasive bronchial lesions are identifiable as areas with decreased autoflourescence [66]. Using this technology experienced bronchoscopists can improve their sensitivity in detecting high-grade dysplasias and CIS by at least three-fold in comparison to white light bronchoscopy [65]. This improvement in sensitivity is associated with a slight but statistically significant decrease in specificity. The overall sensitivity for detecting these lesions by LIFE bronchoscopy is ~70%, and thus is not great enough to warrant routine employment of LIFE bronchoscopy in screening protocols. However, LIFE bronchoscopy will undoubtedly play an important role in validating proposed molecular screening assays and in verifying the presence of these high-risk lesions in patients identified through such screening procedures.

Biomarkers for chemoprevention studies

Chemoprevention studies in the lung are hampered by a lack of intermediate markers for treatment evaluation. Through the use of LIFE bronchoscopy it is now possible to diagnose preneoplastic lesions with a higher sensivity than previously [65]. The WHO system of classification for bronchial epithelial lesions includes eight morphological categories: normal, reserve cell hyperplasia, squamous metaplasia, mild, moderate and severe dysplasia, carcinoma *in situ* and invasive carcinoma. A study designed to validate

this classification scheme evaluated interobserver variability between 10 internationally recognized pulmonary pathologists who independently classified a set of images of bronchial lesions distributed through the internet [67]. In addition to demonstrating that the distribution of internet images could be used to successfully evaluate interobserver variability, this study also clearly showed a high degree of interobserver agreement between pulmonary pathology experts in employing this classification of bronchial epithelial lesions. An interclass coefficient of 0.90 was found in this analysis (F.R. Hirsch, University of Colorado Helath Sciences Centre, Denver, CO, USA, personal communication).

An analysis of lesions in a high-risk cohort of subjects (patients with a diagnosis of chronic obstructive pulmonary disease and a smoking history >30 pack-yrs) found a difference in baseline morphological changes between former smokers and current smokers. Former smokers were found to have a lower level of dysplastic changes than current smokers [68]. An ongoing University of Colorado Cancer Center study of repeat bronchial biopsies taken from the same site as previously diagnosed bronchial lesions is being performed to validate the role of histological and immunohistochemical analyses as intermediate biomarkers for the evaluation of chemopreventive studies. Preliminary data have shown a statistical correlation between histological changes in the bronchial epithelium and the expression of markers such as Ki67, MCM2 and p53 [68]. This study is evaluating three models for the detection of changes in preneoplastic disease between initial and follow-up bronchoscopies: 1) changes in worst-to-worst diagnoses (*i.e.* the histological diagnosis in initial *versus* repeat bronchoscopy, respectively); 2) changes in average score of biopsy diagnoses (*i.e.* a diagnostic score based on a calculated average for a set of biopsies is compared between initial and repeat bronchoscopies); 3) changes in dysplasia index (*i.e.* fraction of biopsies with any degree of dysplasia as related to total number of biopsies taken at each bronchoscopy). Hopefully such studies will lead to a definition of a response model that can be used as an intermediate end-point in the evaluation of studies of chemopreventive agents.

To conclude, recent advances in the understanding of the molecular biology of premalignant and early invasive lung lesions has helped identify many promising potential markers of early lesions which will lead to new effective screening assays in high-risk populations. Using these assays in conjunction with advances in the radiographical detection of early lung cancer should further improve the ability to detect lung cancer prior to the development of advanced stage disease. By identifying these precursors and early invasive lesions in patients at high risk for the development of primary and secondary lung cancers it should ultimately be possible to reduce the overall mortality associated with lung cancer. Efficacious therapeutic options will be needed to achieve this reduction in the toll associated with this disease. Biomarkers will also be invaluable in evaluating potential treatments for preventing the progression of premalignant and malignant lung lesions. Clinical trials are being planned and are currently underway to evaluate these newly identified diagnostic and therapeutic approaches, and the results are being eagerly awaited.

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