Biomarkers in Cancer Chemoprevention
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Lung cancer: chemoprevention and intermediate effect markers

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Even after smoking cessation, genetic damage in the airways epithelium may lead to focal progression of lung carcinogenesis. Some centres now report as many new lung cancer cases among former smokers as among current smokers. Chemoprevention is a potential approach to diminish the progression of pre-clinical genetic damage. The most intensively studied lung cancer chemoprevention agents are the retinoids, including vitamin A and its synthetic analogues and precursors. While effective in suppressing lung carcinogenesis in animal models, retinoids have failed to inhibit carcinogenesis in human chemoprevention trials with premalignant end-points (sputum atypia, bronchial metaplasia). In trials with lung cancer end-points, administration of retinoids either was ineffective or, in the case of β-carotene, led to greater lung cancer incidence and mortality. In view of these findings, markers of specific retinoid effect (i.e., levels of RAR-β) become less relevant. Other markers of genetic instability and proliferation may be useful for both early detection and potentially as intermediate-effect markers for new chemoprevention trials. Cytological atypia, bronchial metaplasia, protein (hnRNP A2/B1) overexpression, *ras* oncogene activation and tumour-suppressor gene deletion, genomic instability (loss of heterozygosity, microsatellite alterations), abnormal methylation, helical CT detection of atypical adenomatous hyperplasia and fluorescent bronchoscopic detection of angiogenic squamous dysplasia offer great promise for molecular diagnosis of lung cancer far in advance of clinical presentation. These end-points can now be evaluated as monitors of response to chemoprevention as potential intermediate-effect markers.

Chemoprevention

Lung cancer continues to be the leading cause of cancer death in most developed countries. In the United States in 1998, 171 500 new cases and 160 100 deaths maintained lung cancer as the leading cancer killer of both men (exceeding prostate cancer) and women (exceeding breast cancer) (Landis *et al.*, 1998). The persistence of grim lung cancer incidence and mortality figures over three decades demands new approaches to control this disease. Tobacco smoking is generally accepted to be responsible for 85–90% of lung cancer. Yet even after cessation of smoking, unrepaired genetic damage in the airways epithelium may lead to focal progression of carcinogenesis. New cases of lung cancer are now as common among former smokers as in current smokers in the United States (Kurie *et al.*, 1995). Chemoprevention is a potential approach to mitigate the progression of preclinical genetic damage. As originally termed by Sporn *et al.* (1976), chemoprevention is the interruption of the carcinogenic process through application of natural or pharmacological agents.

The potential for lung cancer chemoprevention is built upon two observations: field cancerization and multistep carcinogenesis (Lippman *et al.*, 1994). Slaughter *et al.* (1953) first noted the widespread damage (field cancerization) that persists in the aerodigestive tract of current and former smokers. From completely sectioned autopsy specimens, Auerbach *et al.* (1961, 1979) recognized three progressive grades of histological abnormality in the epithelium of 'uninvolved bronchi' surrounding lung tumours: hyperplasia (an increase in the number of cell rows), metaplasia (loss of cilia) and dysplasia (presence of atypical cells). They also reported an increased frequency of carcinoma *in situ* associated with a higher frequency of

smoking, from 0% in nonsmokers to 11% in men who smoked two or more packs per day. Field cancerization implies that detection of premalignant or malignant lesions in one area of the pulmonary epithelium identifies an epithelium at increased risk of developing neoplastic lesions in other areas (Khuri & Lippman, 2000).

Observation of progressive histopathological abnormality preceding cancer reflects the underlying multistep carcinogenesis. Tumours develop through a series of specific genetic changes in proto-oncogenes and tumour-suppressor genes (Bishop, 1987; Fearon & Vogelstein, 1990; Weinberg, 1989). Changes that cannot be repaired and do not trigger apoptosis may lead to a cellular growth advantage. Many of these genetic changes are acquired before and during the earliest stages of clonal expansion and are retained by daughter cells through the course of carcinogenesis and malignancy. Wistuba *et al.* (1999) observed that increasing severity of histopathological changes in lung squamous carcinoma is associated with a progression of genetic changes (increasing frequency of loss of heterozygosity; LOH). Regions of chromosomal loss are suspected to have contained tumour-suppressor genes, the loss of which would be advantageous for growth. This genetic damage is widespread throughout the airway even in areas of normal-appearing epithelium, and persists long after removal of the insult. If detected during the premalignant period, these genetic changes could serve as markers of carcinogenesis and indicate the need for chemoprevention.

Retinoids, including vitamin A and its synthetic analogues, are the most intensively studied lung cancer chemoprevention agents. Retinoids promote the differentiation of airway epithelium *in vitro* (Jetten *et al.*, 1992) and suppress lung carcinogenesis *in vivo* (Moon *et al.*, 1994). Most effects of retinoids on gene expression are mediated by nuclear retinoic acid receptors RARs (α, β and γ) and retinoid X receptors (RXR α, β and γ), which function as retinoid-activated transcription factors. Like the other members of the steroid receptor superfamily, each of these receptors is thought to bind to specific response elements (retinoic acid response elements, RAREs) which govern the expression of genes and modify post-translational mechanisms that regulate cell growth and differentiation (Chambon, 1996; Mangelsdorf *et al.*, 1994). In the case of all-*trans*-retinoic acid, these post-translational mechanisms also may include degradation of CDK-4 through the ubiquitin–proteasome pathway (Sueoka *et al.*, 1999) and binding of E_2F-4 to (the RB family member) p107 leading to transcriptional suppression (Lee *et al.*, 1998). Reduced RAR-β expression is observed in non-small-cell lung cancer (NSCLC) compared to paired normal tissues. Lotan (1997) found that the messenger RNA (mRNA) expression of RAR-β was suppressed in more than 50% of oral and lung premalignant lesions in individuals without cancer (e.g., oral leukoplakia and squamous metaplasia), in dysplastic lesions adjacent to cancer, and in malignant oral and lung carcinomas. Picard *et al.* (1999) measured reduced RAR-β protein levels in tumour compared to normal tissues resected from 76 NSCLC patients using immunohistochemistry. Decreased expression of RAR-β in lung cancer tissue and cell lines suggests that loss of RAR-β expression may be important in the development of lung cancer (Xu *et al.*, 1997).

Metaplasia and dysplasia end-points

Metaplasia and dysplasia of the bronchial epithelium have been monitored as a general index of progression towards lung cancer. Alternatively, RAR-β expression might be considered a specific index of retinoid activity. Examination of these markers might indicate a chemopreventive effect in advance of clinical lung cancer development (intermediate end-points). Synchronous evaluations of these intermediate end-points, however, do not show similar results. Ayoub *et al.* (1999) examined the effects of 13-*cis*-retinoic acid on both bronchial epithelium and RAR-β expression. Decreased baseline expression of RAR-β mRNA was found in bronchial brushings in 44 (23%) of 188 smokers. These 44 were randomly assigned to receive a placebo or 13-*cis*-retinoic acid (30 mg/day) for six months. While only 18 of 44 (41%, eight 13-*cis*-retinoic acid treated and ten placebo) completed the follow-up bronchoscopy, the 13-*cis*-retinoic acid group showed a significant upregulation of RAR-β expression at the end of 13-*cis*-retinoic acid treatment ($p = 0.001$). Corresponding cytological changes in bronchial brushings were uncommon. Thus, RAR-β expression may be upregulated by 13-*cis*-retinoic acid treatment even in the absence of cytological improvement. Xu *et*

al. (1999) evaluated the effects of 13-*cis*-retinoic acid on bronchial mucosal biopsies of heavy smokers and found a significant increase in RAR-β mRNA in the biopsies of the treated group after six months of treatment without a reversal of squamous metaplasia.

Khuri and Lippman (2000) and Siegfried (1998) have summarized the published results of randomized clinical trials of lung cancer chemoprevention. Lung premalignancy (sputum atypia or bronchial metaplasia) was the end-point of five trials (Table 1). All three randomized trials that studied the effect of retinoids on lung premalignancy in smokers gave negative results. In their small (n = 150), placebo-controlled trial of etretinate to reverse sputum cytology metaplasia, Arnold *et al.* (1992) found that metaplasia was reversed in 32% of etretinate-treated subjects and 30% of placebo subjects. In a placebo-controlled trial of isotretinoin (n = 87), Lee *et al.* (1994) found similar reductions in metaplasia index regardless of treatment (54% isotretinoin vs. 60% placebo). Smoking cessation during this trial showed a better correlation with reduction in metaplasia index than did chemopreventive treatment. In a similarly designed trial of fenretinide in 68 subjects, there was also a negative result (Kurie *et al.*, 1999).

Sputum atypia was the premalignancy end-point of two randomized studies of chemoprevention. Treatment with β-carotene (50 mg per day) plus retinol (25 000 IU on alternate days), led to no significant reduction in the prevalence of sputum atypia or in cytological progression in 755 asbestos workers (McLarty *et al.*, 1995). Although Heimburger *et al.* (1988) reported a significant improvement in sputum atypia in a folic acid–vitamin B_{12} treatment group after a four-month, randomized, placebo-controlled trial (n = 73), a reanalysis of these data using standard analytical methods found no significant difference in sputum atypia between the placebo and treatment groups (Lippman *et al.*, 1994).

Primary lung cancer end-point

Two large randomized trials sponsored by the US National Cancer Institute have evaluated chemoprevention of primary lung cancer. In the Alpha-Tocopherol, Beta-Carotene (ATBC) Prevention Study, α-tocopherol and β-carotene were examined in a randomized, placebo-controlled factorial

Table 1. Results of randomized aerodigestive clinical/translational trials

Intervention	End-point	Number	Result	Reference/Year
Etretinate	Metaplasia	150	Negative	Arnold *et al.* (1992)
Isotretinoin	Metaplasia	87	Negative	Lee *et al.* (1994)
Fenretinide	Metaplasia	68	Negative	Kurie *et al.* (1999)
β-Carotene + retinol	Sputum atypia	755	Negative	McLarty *et al.* (1995)
Vitamin B_{12} + folic acid	Sputum atypia	73	Negative	Heimburger *et al.* (1988)
β-Carotene	Lung cancer	29 133	Harmful	Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group (1994)
β-Carotene + retinol	Lung cancer	18 314	Harmful	Omenn *et al.* (1996)
β-Carotene	Lung cancer	22 071	Negative	Hennekens *et al.* (1996)
β-Carotene	Lung cancer	39 876	Negative	Lee *et al.* (1999)
Vitamin E	Lung cancer	29 133	Negative	Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group (1994)
Retinyl palmitate	Second primary LC	307	Negative	Pastorino *et al.* (1993)
Retinyl palmitate	Second primary LC	2592	Negative	de Vries *et al.* (1999)
N-Acetylcysteine	Second primary LC	2592	Negative	de Vries *et al.* (1999)

Adapted from Khuri & Lippman (2000)

Table 2. Intermediate effect markers

Marker	Reported in pre-malignant/ malignant specimens	Reference
RAR-β mRNA	Pre-malignant	Lotan (1997), Ayoub *et al.* (1999)
hnRNP A2/B1	Pre-malignant	Tockman *et al.* (1997), Fielding *et al.* (1999)
ras oncogene activation	Pre-malignant	Rodenhius & Slebos (1992), Mao *et al.* (1994a)
p53 deletion	Pre-malignant	Hollstein *et al.* (1991), Mao *et al.* (1994a)
Loss of heterozygosity at 3p, 9p	Pre-malignant	Mao *et al.* (1994b, 1997)
Promoter CpG island methylation	Pre-malignant	Herman *et al.* (1996), Belinsky *et al.* (1998)
Helical CT detection of atypical adenomatous hyperplasia	Malignant Malignant	Henschke *et al.* (1999), Kitimara *et al.* (1999)
Fluorescent bronchoscopy detection of angiogenic squamous dysplasia	Pre-malignant	Lam *et al.* (1993)

design among 29 133 Finnish male cigarette smokers (Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). After 5–8 years receiving either (1) β-carotene (20 mg/day), (2) α-tocopherol (50 mg/day), (3) the combination or (4) placebo, the group receiving β-carotene had a statistically significant 18% increase in lung cancer incidence and an 8% increase in total mortality compared with the placebo group. This result was in sharp contrast with the epidemiological data, which show an inverse association of dietary and serum β-carotene with lung cancer (IARC, 1998). Smokers who consume more fruits and vegetables are reported to have a lower risk for lung cancer, but the nutrients responsible for this protection remain unknown. Carotenoids in fruits and vegetables were postulated to have a protective action by quenching singlet oxygen and free radicals that could lead to lipid peroxidation.

The Beta-Carotene and Retinol Efficacy Trial (CARET) confirmed the harmful effect of supplemental β-carotene upon cigarette smokers (Omenn *et al.*, 1996). CARET was a multicentre, randomized, double-blind, placebo-controlled primary prevention trial in the United States involving a total of 18 314 smokers, former smokers and workers exposed to asbestos. This trial was stopped 21 months early when interim analysis showed that a combination of 30 mg of β-carotene and 25 000 IU of retinol per day had no benefit and may have had an adverse effect on the incidence of lung cancer and on the risk of death. The active treatment group had statistically significant excesses of lung cancer incidence (28%), lung cancer mortality (46%), cardiovascular mortality (26%) and all-cause mortality (17%) compared with the placebo group.

The reasons for the harmful effects of β-carotene supplementation are neither clear nor universal. No significant harmful (or beneficial) effect was observed on rates of lung cancer, total cancer or cardiovascular disease after 12 years of β-carotene supplementation (50 mg, alternate days) among 22 071 males in the Physicians' Health Study (Hennekens *et al.*, 1996). While many physicians were nonsmokers, there were no differences

between current and former smokers in any of these end-points. Similarly, the Women's Health Study found neither positive nor negative effects of β-carotene (50 mg, alternate days) on the incidence of cancer or of cardiovascular disease (Lee *et al.*, 1999). This randomized, double-blind, placebo-controlled trial among 39 876 women aged 45 years or older tested the effects of aspirin, vitamin E and β-carotene in prevention of cancer and cardiovascular disease. The β-carotene component was terminated early after a median treatment duration of 2.1 years in response to the publication of the Finnish and CARET trials. While no mechanism satisfactorily explains the effects of β-carotene on lung cancer, the absence of beneficial effects and the potential for harm suggests that this agent should not be used for chemoprevention (IARC, 1998; Hong, 1999).

Second primary lung cancer end-point

Perhaps as a result of widespread epithelial injury (field cancerization), patients with a successfully resected first primary lung cancer have a high annual incidence (2–5%) of second primary lung cancer (Grover & Piantadosi, 1989). The adjuvant effect of vitamin A (as retinyl palmitate, 300 000 IU per day for 12 months) was evaluated in 307 patients with stage I NSCLC, randomly assigned to treatment or placebo after curative surgery. After a median follow-up of 46 months, the treatment group developed fewer second primary tumours (37% vs. 48%) and showed a significantly greater time to new primary (Pastorino *et al.*, 1993). These encouraging results have been followed by the EUROSCAN trial, the largest chemoprevention study in curatively treated early-stage oral cancer, laryngeal cancer and lung cancer (n = 2595) (de Vries *et al.*, 1999). Initiated by the European Organization for Reseach and Treatment of Cancer (EORTC) in 1988, EUROSCAN was an open-label, multicentre, two-year trial of retinyl palmitate and *N*-acetylcysteine (NAC) administered in a 2×2 factorial design. Preliminary results show no difference between treatment and control arms for second primary tumours of the head and neck and the lung, local/regional recurrence and distant metastases, or long-term survival rates.

In trials designed to look at primary aerodigestive tract end-points therefore, chemoprevention of premalignancy (sputum atypia or bronchial metaplasia) has been unsuccessful and chemoprevention of primary or second primary lung cancer has either been unsuccessful or harmful (Table 1). The intermediate end-point of specific retinoid (13-*cis*-retinoic acid) effect, RAR-β mRNA expression has differed from the more general lung cancer premalignancy markers (sputum atypia and bronchial metaplasia). The absence of benefit from chemoprevention with 13-*cis*-retinoic acid in reducing lung cancer incidence indicates that a marker of retinoid effect does not necessarily provide a good intermediate index of lung cancer chemoprevention.

Lung cancer as secondary end-point

In terms of lung cancer as a secondary end-point, vitamin E and selenium show some promise for chemoprevention. In a placebo-controlled trial to evaluate the effect of selenium (200 μg in brewer's yeast per day) on the incidence of skin basal-cell or squamous-cell carcinomas, Clark *et al.* (1996) found a reduction of second primary lung cancers. Seven dermatology clinics in the eastern United States treated 1312 former non-melanoma skin cancer patients with selenium or placebo for an average of 4.5 years. After follow-up of 8271 person-years, selenium treatment did not significantly affect the incidence of basal-cell or squamous-cell skin cancer. However, compared with controls, patients treated with selenium had significant reductions in total cancer mortality, total cancer incidence and incidence of lung, colorectal and prostate cancers. The trial was stopped early because of the apparent reductions in total cancer mortality and total cancer incidence in the selenium group. No case of selenium toxicity occurred. The potential lung cancer risk of low selenium levels is supported by follow-up data from a Finnish serum bank (Knekt *et al.*, 1998). The selenium levels of 95 cases of lung cancer arising during a 20-year follow-up of 9101 cancer-free individuals were compared with 190 matched controls. After adjustment, Knekt *et al.* (1998) found a significant excess lung cancer relative risk among those in the lowest compared with the highest tertiles of serum selenium levels. The low selenium–lung cancer association was even stronger at lower levels (<5.9 mg/litre) of α-tocopherol.

A slight chemopreventive effect of vitamin E was observed in the α–tocopherol arm of the ATBC

study (Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). In contrast to the significant increase in lung cancer incidence with β-carotene, vitamin E gave no adverse effect on lung cancer incidence, but a trend towards reduction in lung cancer incidence in participants with longer α-tocopherol exposure. The epidemiological dietary and baseline serum α-tocopherol levels from the ATBC study support the protective effect of vitamin E against lung cancer (Woodson *et al.*, 1999), as do data from China. An occupational cohort of Chinese tin miners was followed for development of lung cancer. Although there was no significant overall association between prospectively collected serum α-tocopherol, γ-tocopherol or selenium levels and incidence of lung cancer, results from this study suggest that higher α-tocopherol levels may be protective in men less than 60 years old and in those who do not drink alcohol (Ratnasinghe *et al.*, 2000).

In summary, while natural and synthetic vitamin A metabolites and analogues (retinoids) have been found to suppress head and neck and lung carcinogenesis in animal models, chemopreventive interventions have failed to show inhibition of carcinogenesis in individuals with premalignant lesions and were either neutral or harmful in terms of lung cancer end-points. With no demonstrated effectiveness of retinoid chemoprevention, discussion of markers of specific retinoid effect (i.e., RAR-β) becomes moot.

Intermediate-effect markers

A number of genetic instability and proliferation markers have been proposed as general markers of carcinogenesis. These markers may be useful both for early detection and potentially as intermediate-effect markers for chemoprevention trials.

Abnormal protein (hnRNP A2/B1) overexpression

Sputum cells can now be probed for altered gene expression to assess the preneoplastic state of the airway. During the Johns Hopkins Lung Project (JHLP), we developed an archive of dysplastic sputum specimens and associated clinical data linking specimens to lung cancer outcome. These archived specimens were examined with two monoclonal antibodies (MoAbs 703D4 and 624H12; raised at the US National Cancer Institute) to NSCLC and SCLC cell lines (respectively). Antibody differential display was used to identify biomarkers of lung cancer in archived sputum specimens two years before clinical detection of lung cancer, with a sensitivity of 91% and a specificity of 88% (Tockman *et al.*, 1988).

Monoclonal antibody 703D4 recognizes an epitope of hnRNP A2 and its splice variant, hnRNP B1 (Zhou *et al.*, 1996). The target antigen for 703D4 was purified, leading to detection of three sequences, including one across a site of alternate exon splicing, which all identified a single protein, heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2). The splice variant hnRNP-B1 was a minor co-purifying immunoreactive protein. hnRNPs are members of a family of ribonuclear proteins which are generally thought to regulate the shuttling of nascent RNA transcripts between the nucleus and cytoplasm. The hnRNP A2/B1 family of antigens is frequently observed in transformed bronchial epithelium (Zhou *et al.*, 1996) and its increased expression is associated with a critical phase of fetal lung development for three mammalian systems, suggesting an oncofetal role for this protein (Montuenga *et al.*, 1998).

We are conducting a clinical trial to evaluate the performance of the hnRNP A2/B1 protein as a biomarker for early detection of second primary lung cancer (SPLC) (Tockman *et al.*, 1994). Individuals at risk for SPLC have the highest annual incidence of lung cancer (2–5%) among asymptomatic populations (Grover & Piantadosi, 1989). The Lung Cancer Early Detection Working Group (LCEDWG), thoracic surgeons and medical oncologists from leading medical centres throughout the United States and Canada, and the US National Cancer Institute are collaborating in this trial.

Monoclonal antibody 703D4 binds hnRNP A2/B1 within selected epithelial cells that exfoliate in the sputum (Tockman *et al.*, 1998). In all cells correctly diagnosed by immunocytochemistry, we recognize at least a proplastic morphology. These morphological criteria reflect proliferative changes in nuclear morphology and a level of cytoplasmic immaturity. When such cells bind monoclonal antibody, we consider them sentinel cells for preclinical lung cancer (Tockman *et al.*, 1998). Sentinel cells expressing upregulated levels of hnRNP A2/B1 are found infrequently (1/5000) among cells which normally express low levels of

this protein. By developing a cell-based diagnostic approach, rather than a traditional mass extraction assay, we preserve in these isolated cells the natural upregulated signal compared to background noise.

After the first year of the LCEDWG trial, 13 SPLCs were identified (Tockman *et al.*, 1997). The sensitivity and specificity of the hnRNP A2/B1 biomarker for later SPLC were 77–82% and 65–81%, respectively. Among the cases called positive by immunocytochemistry and image cytometry, 67% developed SPLC within one year. This diagnostic accuracy exceeds that commonly found in cancer screening tests with prostate-specific antigen (PSA) (Tockman, 1996). Sueoka *et al.* (1999) and Fielding *et al.* (1999) have independently confirmed that this epitope can be used to detect preclinical lung cancer. More than 6000 individuals have been screened with this biomarker in ongoing clinical trials in North America, the United Kingdom, China and Japan. Detection of hnRNP A2/B1 overexpression in sputum epithelial cells with proplastic morphology appears to be the basis of a cytotest that could be the basis for a strategy of preclinical lung cancer diagnosis.

Specific oncogene activation (ras) and tumour-suppressor gene deletion (p53, 3p, 9p)

Three closely related genes, H-*ras*, N-*ras* and K-*ras* make up the *ras* family of oncogenes. The highly conserved 21-kDa protein products of these genes are important signal transduction elements which participate in cell cycle regulation, controlling proliferation. Mutation of the *KRAS2* oncogene is one of the most commonly occurring genetic lesions in colorectal cancer (Bos *et al.*, 1987), and is frequently seen in lung cancer (Rodenhuis & Slebos, 1992). Using the JHLP archive of preclinical sputum linked to tumour outcome data, we have demonstrated that specific mutations can be detected in non-malignant sputum specimens in advance of clinical lung cancer (Mao *et al.*, 1994a).

In this pilot study, we selected 15 participants in the JHLP with no malignancy in sputum cytology who went on to develop adenocarcinoma or large-cell carcinoma of the lung. These histological cell types were selected because they have a higher incidence of K-*ras* mutations (30%) than other lung tumours (Rodenhuis *et al.*, 1988). We also looked for *p53* gene mutations because these are among the most common genetic alterations in lung cancers (and other cancers) (Takahashi *et al.*, 1989; Hollstein *et al.*, 1991). The first exon of K-*ras* or exons 5–8 of the *p53* gene were amplified by polymerase chain reaction (PCR) from DNA extracted from the paraffin-embedded primary lung tumour. After cloning, the K-*ras* gene was sequenced to detect mutations. Tumours not containing K-*ras* mutations were sequenced for *p53* mutations to detect tumour-specific markers. Once mutations specific for each tumour were identified, oligonucleotide probes were prepared, specific for the wild-type sequence or individual mutant K-*ras* and *p53*. These probes were hybridized to sputum DNA that had been amplified by PCR, cloned into a phage vector and transferred to nylon membranes. Ten of the 15 patients had primary tumours which contained either a K-*ras* or a *p53* gene mutation. Identical mutations were detected in nonmalignant sputum cells from 8 of 10 patients who had tumours containing oncogene mutations. Patients whose tumours did not contain mutations and control patients without cancer were negative for sputum mutations by this assay.

This study demonstrated that 8 of 15 patients (53%) with adenocarcinoma or large-cell carcinoma of the lung had detectable mutations in sputum cells from 1 to 13 months before clinical diagnosis. The identification of specific gene abnormalities is less sensitive than the protein marker described above and its applicability is further limited by the need to know the specific mutation sequence with which to probe the sputum specimens. In this pilot study, the mutation sequence was determined from the resected tumour. This approach is obviously not practical for screening undiagnosed individuals. However, with future advances in gene chip technology, it may become feasible to probe for all possible mutations of common oncogenes and tumour-suppressor genes in sputum specimens of asymptomatic individuals.

Genomic instability (loss of heterozygosity, microsatellite alterations)

Microsatellite markers are small repeated DNA sequences found in the introns (non-coding regions) of a gene. PCR amplification of these repeat sequences provides a rapid method for assessment of loss of heterozygosity (LOH) and facilitates mapping of tumour-suppressor genes

(Nawroz *et al.*, 1994; Ruppert *et al.*, 1993). Yet microsatellites can provide additional information. Expansions or deletions of these repeating elements are called microsatellite alterations. These alterations, acquired during division of a single transformed cell, are passed onto daughter cells during clonal expansion. Since they are not transcribed, microsatellite alterations provide no growth advantage to the cell. However, their detection in histological specimens is equivalent to the detection of neoplastic (clonal) cell populations. Although detection of microsatellite alterations does not indicate the specific genetic change in the tumour, detection of clonal cell populations might serve as a marker for use in cancer screening (Mao *et al.*, 1994b).

Widespread microsatellite instability was first reported in colorectal tumors (Peinado *et al.*, 1992). In hereditary nonpolyposis colorectal carcinoma (HNPCC), mutations of mismatch repair genes are probably responsible for microsatellite alterations at multiple locations in the genome (Leach *et al.*, 1993). However, in non-HNPCC-associated tumours, including lung cancer, there is not a similar widespread loss of mismatch repair, indicating that another, as yet unknown, mechanism is responsible for somatic alterations of repeat sequences (Merlo *et al.*, 1994).

The pattern of microsatellite alterations and LOH may be specific for different types of cancer. The high incidence of these changes on chromosomes 3, 5, 8, 9, 10, 11, 17 and 20 has been described in lung cancer specimens (Merlo *et al.*, 1994; Xu *et al.*, 1997b), although the role of these changes in carcinogenesis is not yet known. Perhaps it is the cumulative effect of these genetic injuries that is important. We have already shown that microsatellite alterations are clonal markers for detection of human lung cancer, and microsatellite alterations at selected loci can be recognized in sputum cells before clinical lung cancer (Mao *et al.*, 1994b). When microsatellite alterations occurred at more than one locus, there was a significant association with hnRNP A2/B1 overexpression (Zhou *et al.*, 1999). We tested 41 paired tumour and normal DNA specimens from NSCLC patients surgically resected at the Moffitt Cancer Center. Eleven di- and tetranucleotide repeat markers were selected for their high frequency of loss (LOH) or alteration in lung cancer. In 41 paired tumour/normal samples, 19 patients (46%) had more than two loci of microsatellite instability (loss or alteration), while 13 out of 41 patients (32%) had only one locus of microsatellite instability. The total frequency for microsatellite alteration was 78%, suggesting that this panel of markers may have a sensitivity comparable to that of hnRNP protein markers for detection of lung cancer. We are now evaluating whether this performance is maintained in sputum specimens of high-risk individuals.

Abnormal methylation

The *p16* gene is located on the short arm of chromosome 9 (at 9p21) and is frequently mutated or inactivated in tumours and cell lines derived from lung cancer (Hamada *et al.*, 1998; Shapiro *et al.*, 1995). This gene codes for a protein that binds to the cyclin-dependent kinases 4 or 6 (CDK4 or CDK6) and prevents the kinase from phosphorylating (activating) cyclin D1. When phosphorylated, the activated cyclin D1 phosphorylates the retinoblastoma protein to allow release of E_2F transactivators and progression through the cell cycle (Lukas *et al.*, 1995). *p16*, therefore, acts as a tumour-suppressor gene, inhibiting mitosis and cell proliferation. Downregulation or loss of *p16* expression could contribute to the loss of cell-cycle control and provide a cellular growth advantage. Mao *et al.* (1997) have shown that nearly two thirds of former smokers show some genetic alteration in their bronchial cells. The frequency of LOH at the tumour-suppressor sites 9p21(*p16*) and 17p13(*p53*) was nearly the same for smokers and for former smokers. In contrast, lifetime nonsmokers showed no LOH at 9p21, suggesting that loss of 9p21 sequences might be an early event in the development of lung cancer.

Loss of *p16* expression through gene deletion or expression of an altered protein through gene mutation are established causes of loss of tumour-suppressor function (Shapiro *et al.*, 1995). Merlo *et al.* (1995) have described inhibition of *p16* gene transcription by promoter region hypermethylation and Herman *et al.* (1996) have described a novel PCR assay for detection of this condition. Briefly, the addition of methyl groups to a sequence motif (CpG islands) in the gene promoter region results in failure of gene transcription (Gonzalez-Zulueta *et al.*, 1995). For *p16*, these

sequence motifs start at the promoter and extend into exon (transcribed region) 1α (Merlo *et al.*, 1995). Myohanen *et al.* (1998) have shown that transcriptional repression induced by CpG island methylation could be at least partly reversed by cell culture treatment with the demethylating agent 5-aza-2′-deoxycytidine.

Belinsky *et al.* (1998) have measured hypermethylation of the CpG islands of the *p16* gene in sputum from lung cancer patients and found a high correlation with the early stages of NSCLC. They suggested that detection of *p16* CpG island hypermethylation might be useful in the prediction of individuals who might develop lung cancer. As yet, however, no prospective studies have assessed the performance of the hypermethylation assay on samples from individuals at risk for developing lung cancer.

Helical CT detection of atypical adenomatous hyperplasia (AAH)

A new radiographic approach has been pioneered in the United States and Japan (Kaneko *et al.*, 1996; Henschke *et al.*, 1999) using low-dose, single-breath, helical computed tomography (CT). Henschke *et al.* (1999), in their Early Lung Cancer Action Project (ELCAP), screened 1000 smokers and former smokers aged 60 years and older with CT and detected 27 lung cancers, over 80% of which were stage 1a tumours. However, the histological distribution of these lung lesions was skewed; 78% were adenocarcinoma and a further 11% were mixed adenosquamous cancers. The very small 'ground glass opacities' detected by CT are frequently characterized histologically as atypical adenomatous hyperplasia (AAH), appearing as type II pneumocytes proliferating along the alveolar septa. Kitamura *et al.* (1999) have characterized AAH as alveolar intraepithelial neoplasia. Certain populations of AAH cells exhibit active proliferation, aneuploidy, 3p and 9p deletions, K-*ras* codon 12 mutation and disruption of cell-cycle control, but *p53* gene aberrations are rare and telomerase activation is absent (Kitamura *et al.*, 1999). AAH and AAH-like carcinomas may constitute a population of heterogeneous lesions representing progressive steps towards overt bronchiolo-alveolar lung carcinoma (Kitamura *et al.*, 1995). Although the treatment and prognostic implications of the lesion have not yet been determined, helical CT-detected AAH could be characterized and evaluated as a surrogate end-point biomarker in future chemoprevention trials.

Fluorescent bronchoscopy detection of angiogenic squamous dysplasia

Experience in other epithelial organs (i.e., cervix, oesophagus, colon) has shown that if the neoplastic lesion can be detected and treated at the earliest, intraepithelial stage, the rate of cure can be significantly improved (Anderson *et al.*, 1988; Jiang *et al.*, 1989; Winawer *et al.*, 1993). Lam *et al.* (1998) showed that for the early detection of bronchogenic carcinoma, a combination of conventional white-light bronchoscopy and light-induced fluorescence endoscopy significantly increased the ability of the bronchoscopist to recognize and diagnose small invasive cancers, carcinoma *in situ* and intraepithelial lesions. The treatment and survival implications of fluorescence-detected lesions have not yet been studied.

In one study of 54 patients with known or suspected lung cancer, 15% of patients diagnosed with invasive carcinoma also harboured carcinoma *in situ* (Lam *et al.*, 1993). The relative sensitivity of fluorescence bronchoscopy, with regard to identification of moderate/severe dysplasia or carcinoma *in situ*, was 50% greater than that of conventional white-light bronchoscopy. To our knowledge, no study has evaluated whether lesions detected by fluorescence bronchoscopy have enhanced significance due to altered gene expression or the presence of angiogenic squamous dysplasia.

In the biopsies from fluorescence bronchoscopies of smokers at high risk for lung cancer, Keith *et al.* (2000) observed a lesion consisting of capillary blood vessels closely juxtaposed to and projecting into metaplastic or dysplastic squamous bronchial epithelium, which they termed angiogenic squamous dysplasia. This lesion represents a qualitatively distinct form of angiogenesis in which there is architectural rearrangement of the capillary microvasculature. Genetic analysis of surface epithelium in a random subset of lesions revealed loss of heterozygosity at chromosome 3p in 53% of angiogenic squamous dysplasia lesions. No confirmed *p53* mutations were identified. Compared with normal epithelium, proliferative activity was markedly elevated in angiogenic

squamous dysplasia lesions. The lesion was not present in biopsies from 16 normal nonsmoker control subjects. While neither the treatment nor prognostic implications of this lesion have been determined, it is another potential intermediate-effect marker for trials of chemoprevention.

Conclusion

These potential intermediate-effect markers offer great promise to establish a molecular diagnosis of lung cancer far in advance of clinical presentation and to follow the response to chemoprevention. Any or all of these tests could be incorporated into the routine management of individuals at risk of developing primary or second primary lung cancer. However, several issues must be considered before these tests are ready for clinical application. First, test performance characteristics must be confirmed in prospective trials. For several of these markers, such trials are already in progress. Second, a management and intervention strategy must be developed that will be appropriate to the stage at which lung cancer is diagnosed. The ability to detect lung cancer at the stage of clonal expansion, well in advance of malignant invasion of the basement membrane, suggests that a non-invasive chemoprevention approach might be the primary therapeutic intervention in such cases. Preliminary studies of chemopreventive agents are now under way at the US National Cancer Institute. Several of these agents could be delivered by inhaler to place a maximum dose directly on the transformed epithelium. The next stage will be clinical trials to evaluate combined diagnostic and therapeutic approaches to assess their impact on the incidence of clinical lung cancer.

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