Journal of Cellular Biochemistry 25S:177–184 (1996)

Clinical Detection of Lung Cancer Progression Markers

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Abstract Lung cancer is the leading cause of cancer-related deaths in western countries. The prognosis for patients with lung cancer depends primarily on the stage of the tumor at the time of clinical diagnosis. New understanding of tumor biology has turned attention away from detection of clinical lung cancer, usually metastatic at presentation, toward recognition of genetic and protein markers which precede malignancy. Mutations of four types of genes contribute to the process of epithelial carcinogenesis by modifying control of cell growth. Examples of three of these changes have been detected in pre-malignant sputum, and validated in subsequent tumor. We have identified gene products (tumor associated and differentiation protein antigens), mutations of *k-ras* and *p53*, and *microsatellite* alterations as potential markers of subsequent malignancy.

We consider the morphologic progression seen in archived sputum cells as the paradigm of neoplastic development in the lung. Although the NCI collaborative trials had shown that this progression is not recognized sufficiently often (sensitive) to be useful for lung cancer screening, this progression may be used to assess the timing of gene and peptide markers of carcinogenesis. Previous work has shown that at the time Johns Hopkins Lung Project sputum cells express moderately atypical metaplasia, 53% (8/15) of sputum specimens expressed common (codon 12) *k-ras* or (codons 273 or 281) *p53* mutations. Other investigators have reported that earlier morphologic changes (metaplasia) accompany 3p and 9p losses of heterozygosity. These observations suggest that 3p and 9p loss likely precede *k-ras* or *p53* mutations. Our preliminary data demonstrate that over-expression of a 31 kD tumor associated antigen recently purified, sequenced, and identified as heterogeneous nuclear ribonucleoprotein (hnRNP) A2 (with cross reactivity to splice variant B1), is expressed in most lung cancer cases before any morphologic abnormality. Comparison of the accuracy of this marker with sputum cytology will determine its value for early lung cancer detection. Preliminary evidence confirms this marker greatly improves the accuracy of standard sputum cytology for detection of lung carcinogenesis. Clinical intervention trials must be undertaken to determine whether modulation of hnRNP overexpression is useful as an intermediate endpoint for chemoprevention. J. Cell. Biochem. 25S:177–184.

Key words: lung cancer; progression markers; sputum cells

The sequence of genetic events which underlies the initiation and promotion of cancer is becoming more clearly understood [Bishop, 1987]. Fearon and Vogelstein [1990] were among the first to connect the acquisition of successive genetic alterations to morphologic transformation and tumor progression. Mutations of four types of genes seem to underlie the process of epithelial carcinogenesis, leading to altered control of cell growth. (Proto)-oncogenes are essential to the normal physiology of the cell, often regulating the balance between maturation and growth. A common mutation of the *ras* oncogene, for example, a single mutation in exon 1 (at codons 12 or 61) leads to an inability of the usual mechanism (GTP hydrolysis) to down regulate (turn off) the mutant ras protein, shifting the resting cell from differentiation (during G_0 or interphase) toward proliferation (advancing the cell cycle toward mitosis) [Feig, 1993]. For this reason, oncogene activation has been likened to a sticking "accelerator" for carcinogenesis. The daughter cells which result from oncogene activation form a clone with a growth advantage over adjacent epithelial cells.

A second class of genes, the tumor suppressors, are inactivated during cancer progression by the loss of one or more specific alleles (or

Presented at Prospects for Chemoprevention in Cohorts with Cancer Risk Markers, Tidewater Inn, Easton, Maryland, October 5-7, 1995.

Contract grant sponsor: Chemoprevention Branch, DCPC, National Cancer Institute Grant, contract grant number 1P50 CA58184-01.

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Received 12 February 1996; Accepted 20 September 1996

locations on the gene) [Hartwell and Kastan, 1994]. Following Knudson's hypothesis, mutational inactivation of the first of two copies during somatic tumor development is followed by mutation or loss of the second allele [Knudson, 1971; Stanbridge, 1990]. Loss of both tumor supressor gene alleles may remove the regulatory "brakes" holding the cell at rest in interphase (G_0) and allow progression through the proliferation cycle. For example, DNA damage normally leads to an increased production of *p53* tumor supressor gene protein, resulting in either cellular arrest in G_1 or apoptosis (cell death) [Hartwell and Kastan, 1994]. Mutant p53 protein cannot fulfill these inhibitory functions. The *p53* tumor-supressor gene is found to be mutated in a wide variety of cancers including those of the lung [Takahashi et al., 1989], breast [Varley et al., 1991], esophagus [Hollstein et al., 1990], liver [Bressac et al., 1991], bladder [Sidransky et al., 1991], ovary [Marks et al., 1991], brain [Sidransky et al., 1992], as well as almost every other tumor type [Nigro et al., 1989]. The 393 amino acids of the p53 protein are arranged in domains which have been conserved through evolution at sites where this protein binds to DNA. During carcinogenesis, several of these DNA binding domains become "hot spots" for mutation. The spectrum of these *p53* mutations differs in various tumor types and by smoking history. The *p53* tumor suppressor gene has been identified as one (along with *p16*) of the most commonly mutated genes in human cancers [Greenblat et al., 1994].

The third class of genes which may be mutated during carcinogenesis are the DNA repair genes (excision repair [Sancar, 1994] and mismatch repair [Modrich, 1994]). In excision repair, phosphodiester bonds are hydrolyzed on either side of the damaged nucleotide, creating an oligonucleotide carrying the damage. The excised oligonucleotide is released, and the resulting gap is filled in using the complementary DNA strand as a template. In mismatch repair, the insertion of incorrect bases into newly synthesized DNA is recognized and corrected. Cells with damaged DNA are normally held at cell cycle checkpoints and prevented from replicating until the damage is repaired. Transformation of normal cells into cancer cells is facilitated by loss of coordination between genome repair and the cyclin-dependent kinases and checkpoint controls responsible for progression through the cell cycle [Hartwell and Kastan, 1994].

In some hereditary tumors, the Lynch Syndrome [Leach et al., 1993] for example, failure of normal mismatch repair leads to instability of the genome resulting in single base pair mismatches and small displaced loops that can occur randomly through slippage during replications of repeat regions [Modrich, 1994]. These repeat regions are called "microsatellites." Occasional specific, rather than random microsatellite alterations are common in a variety of acquired human tumors [Mao et al., 1994]. Microsatellites occur between transcribed DNA sequences and therefore offer the cell no growth (or survival) advantage. When DNA is amplified by the polymerase chain reaction (PCR), the appearance of microsatellite alterations indicates the presence of multiple cells (a cell clone) bearing an identical allelic change. Thus microsatellites, which have been found to be altered in a tissue-specific pattern, may have a role in detecting populations of neoplastic cells which have failed localized genomic mismatch repair.

Fourth, mutations in genes which encode cell cycle checkpoints have been found to increase genetic instability and may play a role in carcinogenesis. Hartwell and Kastan [1994] have recently described a model in which activation of cyclin-CDK (cyclin dependent kinase) complexes are required for progression through the successive steps of the cell cycle. Mutation of genes that control the activation of these complexes may result in loss of "checkpoint" function. For example, progression from G_1 into S phase (synthesis of new DNA) requires Rb protein, may be partially dependent on *p53* and probably requires the 9p21 protein, p16 Hartwell and Kastan, 1994].

Each of these mutations presumably arise from the initiating action of a carcinogen and persist in the DNA due to failure of repair prior to cell division. Examples of three of these four classes of genetic alterations has been detected in the pre-malignant sputum. Morphological, protein, and gene markers of neoplastic transformation have been detected in exfoliated epithelial cells of the sputum preceding (by years) the the development of clinical malignancy. Selecting as most valid, those markers expressed both by the archived sputum cells with earliest morphological changes and also expressed in subsequent tumor, we have identified gene products (tumor associated and differentiation protein antigens) [Tockman et al., 1988], mutations of k-ras and p53 [Mao et al., 1994a, and

microsatellite alterations [Mao et al., 1994b] as potential markers of subsequent malignancy.

Using the morphologic changes seen in archived cells, we consider the changes from normal to regular metaplasia, to slight, moderate, and marked dysplasia and finally to neoplasia as the steps of neoplastic progression. Although the NCI collaborative trials had shown that this progression is not recognized sufficiently often (sensitive) to be useful for lung cancer screening, this progression may be used to assess the timing of gene and peptide markers of carcinogenesis. Our previous work has shown that at the time sputum cells express moderately atypical metaplasia, 67% (10/15) of tumor specimens and 53% (8/15) of sputum specimens expressed common (codon 12) k-ras or (codons 273 or 281) p53 mutations [Mao et al., 1994b]. Kishimoto et al. [1995] have reported that hyperplasia (metaplasia) is the earliest epithelial lesion showing 3p loss of heterozygosity in four of seven cases (57%), the remainder showing more advanced morphological change. These investigators also reported that hyperplasia was the earliest lesion in only two of seven (28%) with 9p LOH. These observations suggest that 9p loss might be a later event in the progression toward lung cancer than 3p loss, but earlier than k-ras or p53 mutations. Our preliminary data demonstrates that over-expression of a 31 kD tumor associated antigen recently identified as hnRNP A2/B1 [Zhou et al., in press], is expressed in most cases, prior to any morphologic abnormality. These genetic and related protein markers are now being evaluated in sputum and tumor specimens from lung cancer patients. Preliminary evidence for these gene and protein alterations in sputum preceding lung cancer now raises great enthusiasm for their trial as markers of lung carcinogenesis.

The first of these markers to enter clinical trial is hnRNP overexpression. An archive of sputum and tumor specimens from the collaborative Early Lung Cancer Detection trial conducted at Johns Hopkins (the Johns Hopkins Lung Project, JHLP), enabled the selection of hnRNP as one of two potential markers of subsequent malignancy from among those under consideration by investigators at the National Cancer Institute [Rosen et al., 1984; Burd and Dreyfuss, 1994]. Although the role of this particular molecule in neoplastic progression is unknown, these RNA binding proteins are responsible for the post-transcriptional regulation of gene expression by capping, splicing, polyadenylation, and transportation of mRNAs [Feig, 1993]. This class of proteins has great potential to modulate both signaling and transcription. Over-expression of hnRNP is detectable by a murine IgG_{2b} monoclonal antibody (Mab 703D4) [Mulshine et al., 1983], labeled with a double-bridged avidin-biotin complex [Gupta et al., 1985], and quantified by dual-wavelength image cytometry [Tockman et al., 1993].

We have previously reported the results shown from the Johns Hopkins specimens collected and archived an average of two years before the development of clinical lung cancer (Table 1). Satisfactory sputum specimens from 22 male cigarette smokers who later developed lung cancer were probed for hnRNP expression. Twenty of 22 cases demonstrated epitope binding (sensitivity 91%), 19 of 20 overexpressed hnRNP. Of 40 satisfactory control sputum specimens, 35 showed no hnRNP overexpression (specificity 88%).

To replicate these preliminary results and establish a mechanism to evaluate additional early lung cancer diagnostics, we have initiated two prospective clinical trials at Johns Hopkins. An 11-center trial 'The Early Detection of Second Primary Lung Cancers by Sputum Immunostaining' (ECOG #E5593/SWOG #9437) was begun by the Lung Cancer Early Detection Working Group (LCEDWG), with 11 participating surgical oncology programs [Tockman et al., 1994]. These centers include Cleveland Clinic Foundation, University of Colorado Group, Illinois Cancer Council, Johns Hopkins Hospital, M.D. Anderson Cancer Center, H Moffitt Cancer Center, Hospital Laval (Quebec), Memorial Sloan-Kettering Cancer Center, Mt. Sinai Hospital (Toronto), University of California at Los Angeles, and University of Southern California.

Detection of Subsequent Lung Cancer by Mab Staining of Sputum Cells

	Cancer	No Cancer	Total
Satisfactory			
Stain (+)	20	5	25
Stain (−)	2	35	37
Subtotal	22	40	62
Unsat. specimens	4	3	7
Total	26	43	69

Sensitivity: 91%, Specificity: 88%, Accuracy: 88.7%, OR: 70 (95% CI 10.5–298).

This first trial tests the efficacy of hnRNP overexpression compared to routine Papanicolaou staining of sputum among individuals at high risk for second primary lung cancer (SPLC) [Tockman et al., 1994]. The lifetime incidence of a second primary, defined as a second lung cancer of different histology, is over 10% in these patients with an annual incidence of 1–5% depending on the subgroup [Grover and Piantadosi, 1989]. This is a significantly higher rate than even the heaviest smoking populations and offers a unique laboratory for the study of early detection and chemoprevention.

The LCEDWG/SPLC study design (Fig. 1) provides that individuals who have had a successfully resected surgical Stage I, non-small cell lung cancer, be enrolled after informed consent to undergo an annual induced sputum examination. Each specimen is Papanicolaou-stained and clinically evaluated according to standard morphologic criteria. Aliquots of the preserved specimen are then immunostained and interpreted, blinded to the morphological interpretation. A clinical evaluation is undertaken for patients whose specimens exhibit neoplastic morphology. Individuals whose specimens show normal morphology are asked to return for annual re-examination. A randomized, placebo-controlled, 13-cis Retinoic Acid chemoprevention trial forms a side-arm to this study. Using a second randomization, this side-arm addresses the effectiveness of hnRNP overexpression as an intermediate endpoint for 13-cRA therapeutic effect.

Schema

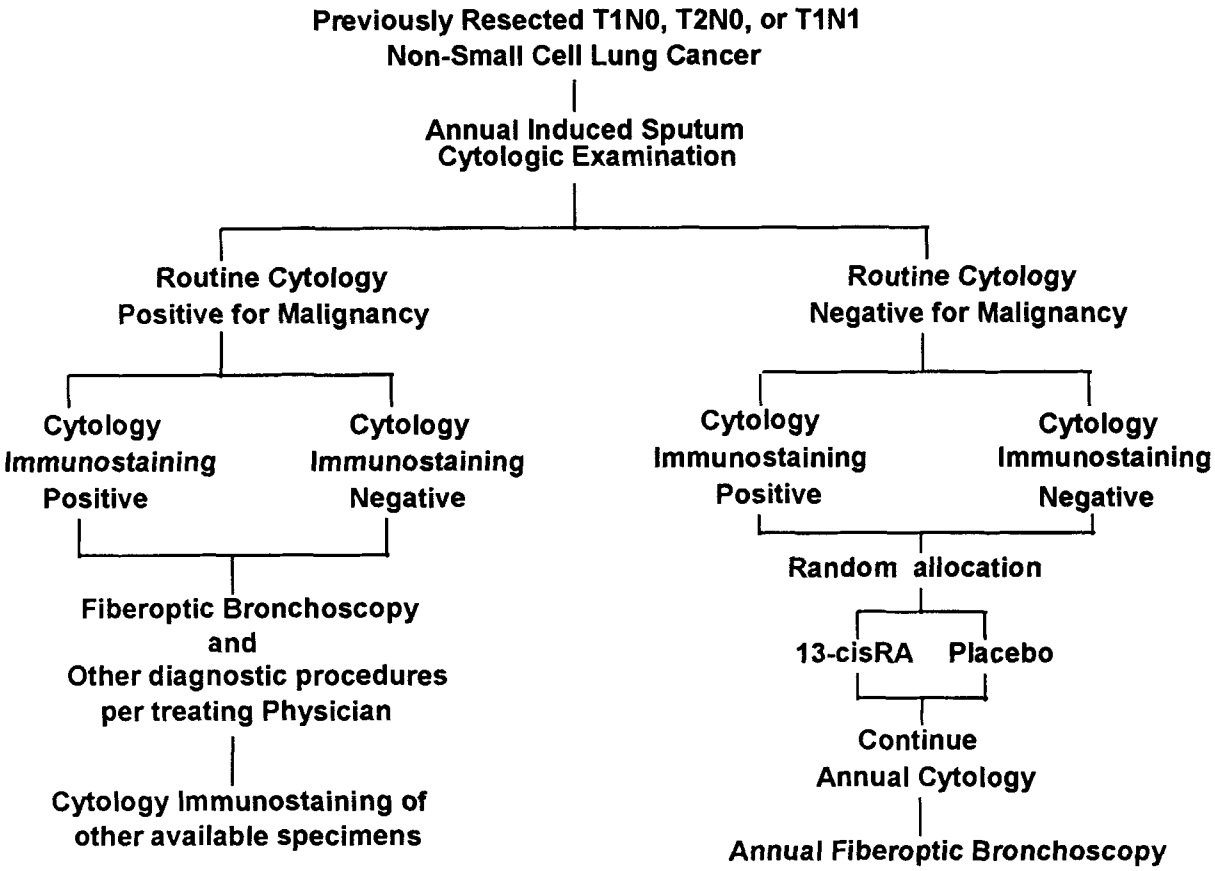

Fig. 1. Study design for the Lung Cancer Early Detection Working Group (LCEDWG) collaborative screening trial; "The Early Detection of Second Primary Lung Cancers by Sputum Immunostaining" (ECOG #E5593/SWOG #9437). Reproduced from Tockman [1994] with permission from the publisher.

In preliminary data from the LCEDWG study, we examine the screening results from 56 individuals who have reached a study endpoint. Twenty-nine individuals developed lung cancer, including 13 second primaries and 16 recurrences. The remaining twenty-seven individuals either died of other causes, or withdrew from the study. Only two of the specimens from these 56 endpoint cases showed any morphologic change, one moderate and one grave atypical metaplasia, both among patients who later developed second primary lung cancer. None of the recurrences showed any morphologic change. In fact, out of the total 29 lung cancers, only 6% could be detected by routine morphology.

The quantitative interpretation of hnRNP overexpression was conducted by immunocytometry using a computer-assisted imaging program (Table 2) [Tockman et al., 1993; Tockman, 1996]. A single lot of monoclonal antibody to hnRNP (designated 703D4) was purified from mouse ascites. The purified antibody was applied to cytospin slides of each patient's specimen and positive control slides. For negative controls, the primary antibody was replaced by a similar protein concentration of mouse IgG_{2b} nonimmune serum. ATCC human bronchogenic cancer cell lines HTB58 (squamous cell cancer) and Calu-3 (adenocarcinoma) were mixed with normal sputum, preserved in Saccomanno's and used as controls. Positive and negative controls were stained and analyzed with each run. All specimens were double bridged and stained with biotinylated diaminobenzidine and counterstained with hematoxylin.

Light intensity transmitted through the immunostained sputum specimens is evaluated at two frequencies. The diaminobenzidine-stained cytoplasm and hematoxylin-stained nuclei have complementary transmission maxima and minima (at 600 nanometers and 510 nm, respectively). At 600 nm, the cytoplasm of a positively staining cell appears relatively translucent, while at 510 nm the cytoplasm appears opaque. Each image was standardized, background-subtracted and shading corrected. Twenty-one morphologic and densitometric factors are measured during analysis. The densitometric features which quantify the cytoplasmic overexpression of hnRNP explain approximately 75% of the variance discriminating individuals who developed cancer from those that did not (Fig. 2) [Tockman, 1996].

Method for Quantitative Immuno-cytometry

- Mouse IgG Mab (703D4) applied to patient specimens
  - ATCC HTB58, CALU3 positive controls
- Non-immune mouse serum applied to neg controls
- All specimens double-bridged
  - Stained with biotinylated DAB
  - Hematoxylin counterstained
- Atypical epithelial cells imaged at 100×
  - Transmitted light at 600 nm, 510 nm
- Calibrated, shading-corrected images evaluated for densitometry, morphometry

Preliminary results show that hnRNP overexpression is positive in 77% (10 of 13) of the LCEDWG second primaries. Of the 27 who have not developed a second cancer, 22 were correctly classified, for a specificity of 82%. This group, of course, is composed entirely of high-risk individuals who will be followed for the next several years, some of whom will likely develop cancer. Nevertheless, even at present, the sensitivity of hnRNP detection exceeds routine sputum cytology at least three-fold and is similar to that of the widely used prostate-specific antigen (PSA) screening test, while the specificity is considerably higher [Lankford et al., 1995].

In a second trial, the frequency of hnRNP overexpression is compared with morphologic changes in exfoliated sputum epithelial cells from Yunnan Tin Corp. underground miners (YTC). The YTC miners are a community dwelling population of tobacco smokers, industrially exposed to radon and arsenic, with an average annual lung cancer incidence of 1% [Qiao et al., 1989; Yao et al., 1994]. Among the 45,000 employees of the YTC, annual radiographic and cytologic screening is offered to the 7,000 at greatest risk. Entry criteria include both active and retired male miners, who are age 45 years or greater, with at least 10 years of underground employment. Overall, the annual lung cancer incidence among these miners is 1.2%. Each year, 70 to 80 cases of lung cancer develop in this group.

Using the methods described above, the sputum specimens from each case plus a randomly selected, age-matched noncancer member of the cohort are reviewed for premalignant morphologic changes as well as overexpression of the hnRNP antigen. Preliminary results from this study have shown that among the first 47 cases of lung cancer, 43 (92% sensitivity) were de-

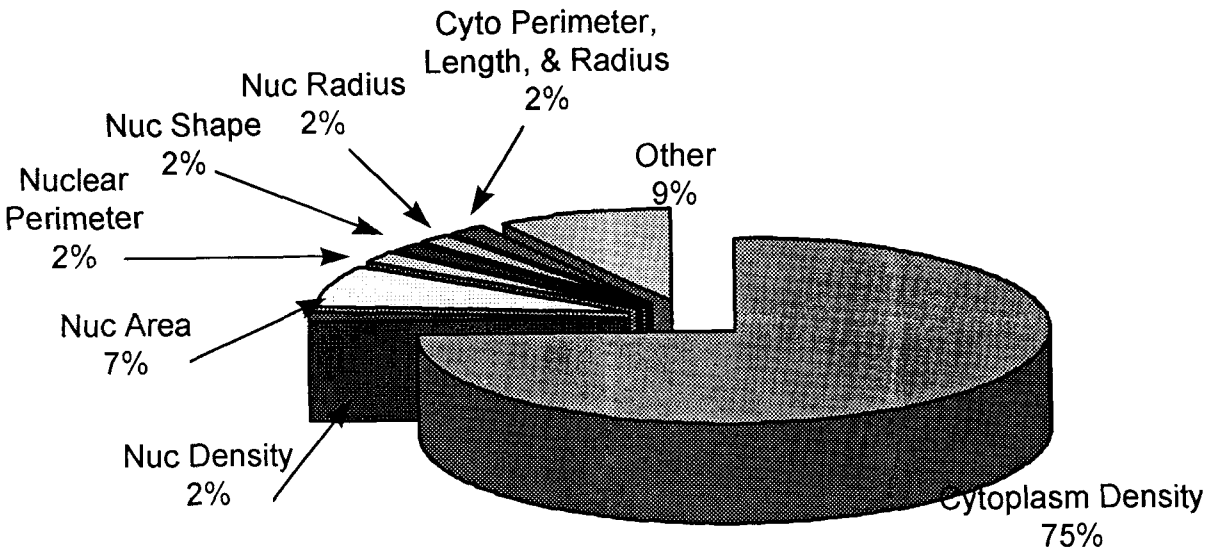

Fig. 2. Cytology specimens are classified as "cancer" or "non-cancer" by a discriminant function algorithm. Compared to the morphologic features of the cell, which individually explain only 2–7% of the difference (variance) between cancer and non-cancer cells, the overexpression of hnRNP 31 kD antigen (cytoplasm density) explains 75% of the difference.

tected by hnRNP overexpression, a level similar to that of the other studies.

In summary, overexpression of the 31 kD hnRNP antigen detected by a murine IgG_{2b} monoclonal antibody (Mab 703D4), labeled with a double-bridged avidin-biotin complex and quantified by dual-wavelength image cytometry has been evaluated as a marker of lung cancer development in the prospectively collected, archived sputum specimens from three separate studies (one completed, two ongoing). Using computer assisted, dual-wavelength image cytometry plus immunostaining, preliminary data from the two ongoing clinical trials continues to demonstrate high levels of marker expression in premalignant sputum of lung cancer cases with reasonable accuracy among controls.

These findings suggest that detection of carcinogenesis could follow a progression of screening tests from earliest and most sensitive (such as overexpression of hnRNP) to most specific (a combination of oncogene mutations and suppressor gene losses of heterozygosity). This detection strategy would have the advantage of speed and low cost, but must first be proven along with a suitable intervention to reduce lung cancer mortality in clinical trial.

As we begin to visualize successful early detection methods on the horizon, we must consider how the early detection results can be used by clinicians to improve the outcome of lung cancer patients. It is critical to consider therapies appropriate for the stage of lung cancer detected. Detection of transformed, but premalignant cells raises the potential to modify the biology to inhibit progression toward malignancy and permit therapies less invasive than surgery. Vitamin A and retinoids are strong inhibitors of epithelial cancer promotion and progression in experimental carcinogenesis. Pastorino and his European colleagues [1993] recently reported a favorable adjuvant effect of high-dose vitamin A on 307 patients who underwent curative stage 1 non-small-cell lung cancer. Following surgery, patients were randomly allocated to receive either retinol palmitate (300,000 IU daily for 12 months) or no treatment. After 46 months of follow-up, 37% of treated patients developed recurrence or second primary, compared to 48% of controls. Clinical investigation would be required to deter-

mine if experimental approaches such as 13-cis Retinoic Acid (noted earlier) or retinol palmitate therapy could be employed to eliminate the bronchial source of marker-bearing cells.

The challenge to public health professionals is to design efficient studies to identify and validate genetic markers as intermediate cancer endpoints. Second, an infrastructure must be developed to allow basic and applied scientists to collaborate in the development of therapies appropriate to the stage of cancer which will be detectable by gene and peptide markers. Finally, these are world-wide health problems and national resources are finite. It is prudent that international mechanisms be explored to conduct the early detection and intervention trials which must be done to allow this exciting science to translate into better health for mankind.

ACKNOWLEDGMENTS

This work was sponsored by Chemoprevention Branch, DCPC, National Cancer Institute Grant 1P50 CA58184-01. I acknowledge the assistance of Y. Erozan, P. Gupta, T. Zhukov, W. Zhou, and Y. Qiao in the conduct of this research and I. Avis and J. Mulshine for provision of Mabs 703D4 and 624H12.

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