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Molecular Epidemiology of Lung Cancer and the Modulation of Markers of Chronic Carcinogen Exposure by Chemopreventive Agents

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Abstract Chronic inhalation exposure to environmental carcinogens such as polycyclic aromatic hydrocarbons (PAHs), cigarette smoke, 4-aminobiphenyl (4-ABP), ethylene oxide, and styrene is associated with elevations in biomarkers such as DNA adducts, protein adducts, sister chromatid exchanges (SCEs), chromosomal aberrations, gene mutation, and/or oncogene activation. These biomarkers indicate an increased cancer risk for the exposed population, although quantitative estimates cannot be made with certainty. There is convincing epidemiological evidence that the antioxidant and free radical-scavenging vitamins C and E and β-carotene (β-C) protect against cancer of the lung and other epithelial tissues, with somewhat weaker evidence for retinol. Experimental studies demonstrate that these micronutrients are capable of blocking or reducing tumor formation caused by diverse carcinogens. A variety of mechanisms appear to be involved, including suppression of carcinogen activation, enhancement of carcinogen detoxification, induction of cellular differentiation, inhibition of mutagenesis, enhancement of immunologic function, and/or reduction of the formation of carcinogen–DNA adducts, SCEs, micronuclei, and other markers of genotoxic damage. Therefore, we have recently investigated the possible modifying effect of serum vitamins C and E, β-C, and retinol on a number of such biomarkers in a case-control study of lung cancer, and in a cross-sectional study of heavy smokers. Preliminary results indicate an inhibitory effect of certain vitamins on DNA adduct formation. A significant number of human intervention trials are ongoing involving these vitamins. It appears that biomarkers can provide useful intermediate endpoints for assessment of both the mechanisms and the efficacy of chemopreventive agents.

Key words: molecular epidemiology, biomarkers, lung cancer, chemoprevention

Abbreviations: AFB_1, Aflatoxin B_1; 4-ABP, 4-aminobiphenyl; B(*a*)P, benzo(*a*)pyrene; β-C, beta-carotene; DEN, *N*-nitrosodiethylamine; DMBA, 7,12-dimethylbenz(*a*)anthracene; DMN, dimethylnitrosamine; MNU, *N*-methylnitrosourea; MNNG, methyl-*N*-nitrosoguanine.

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Molecular epidemiology attempts to prevent cancer by using biomarkers to identify populations and individuals at risk in time to intervene effectively. Molecular epidemiology can also be used to determine the most appropriate intervention strategies (*e.g.*, reducing or eliminating exposures, targeting chemoprevention to already "at risk" groups or individuals, or a combination) and to monitor their effectiveness. While there are many steps yet to be taken in

the validation of biologic markers for this purpose, the promise for prevention is great. An estimated 80% of cancer is deemed preventable because the causative factors are exogenous rather than inborn or inherent. This does not suggest that genetic factors do not play an important role in influencing individual susceptibility, but that in the absence of external factors (such as carcinogenic exposures related to lifestyle, occupation, and ambient environment) an estimated 400,000 of the annual 500,000 deaths in the U.S. could be averted. With respect to lung cancer, over 100,000 of the 140,000 annual deaths are theoretically avoidable; even a 5% reduction in risk would result in 7,000 fewer deaths per year in this country. While active and passive cigarette smoke and occupational carcinogens are the major identified causes of lung cancer, there is growing evidence that ambient exposures also contribute, alone or in combination with smoking [1,2]. Given the very poor 13% overall survival rate in patients with lung cancer, there is an urgent need for new preventive initiatives in addition to smoking cessation programs.

The purpose of this paper is to assess the extent to which biomarkers can be useful in monitoring the efficacy of chemoprevention in lung cancer. The following areas will be reviewed: (1) molecular epidemiologic and biomonitoring data linking environmental exposure to biomarkers and/or lung cancer in humans; (2) experimental and epidemiologic evidence for a chemopreventive role of micronutrients in lung cancer; (3) molecular epidemiologic data relating to the protective effect of the same micronutrients on biomarkers associated with risk of lung cancer; and (4) recommendations for future molecular epidemiologic chemoprevention studies.

MOLECULAR EPIDEMIOLOGY RELEVANT TO ENVIRONMENTAL CARCINOGENESIS OF THE LUNG

Recent studies, including our own, have explored the relationship between inhalation exposure to known carcinogens such as benzo(*a*)pyrene [B(*a*)P], 4-aminobiphenyl (4-ABP), styrene, and ethylene oxide and various biomarkers shown in Figure 1. As arranged along the continuum between exposure and disease, these markers reflect the biologically effective or interacted dose of carcinogens, the resultant biologic/preclinical effects or the genetic/acquired factors that influence these biologic responses, and presumably risk itself. These studies are briefly summarized in Table I. Each of the biomarkers listed in the table has been markedly elevated in exposed or "at risk" individuals as compared to controls in one or more studies, although results have not been uniformly positive. We found that inhalation exposure to carcinogens had a significant effect on multiple biomarkers [carcinogen–DNA and carcinogen–protein adducts, gene mutation, sister chromatid exchange (SCE), micronuclei, chromosomal aberrations, and alterations in oncogenes] in groups exposed to both active and passive cigarette smoking, workplace contaminants, and ambient air pollution.

For example, we have evaluated biomarker levels in a population environmentally exposed to polycyclic aromatic hydrocarbons (PAHs) and other coal combustion products in the ambient air of Silesia, Poland [2]. Approximately 40 residents from Silesia, an industrialized region characterized by high ambient levels of B(*a*)P (winter: 60 ng/m^3; summer: 15 ng/m^3), were compared to a similar number of residents from a rural area with B(*a*)P levels about tenfold lower. Blood samples collected in both winter and summer months were assayed for carcinogen–DNA adducts by immunoassay and ^{32}P-postlabeling; SCE; chromosomal aberrations; and elevation in serum concentrations of the *ras* oncogene protein product p21. Adducts, SCE, and chromosomal aberrations were all significantly increased in the winter samples from the Silesian group as compared to the winter and/or the summer samples from the control group. The exposed group also showed a doubling in the frequency of p21 overexpression compared to controls, although the difference was not statistically significant. Aromatic DNA adducts were correlated with chromosomal aberrations ($r = 0.29$; $p = 0.05$), linking the molecular dose of these pollutants with a biomarker of genetic effect. Of the micronutrients, only retinol could be evaluated; it was not inversely correlated with levels of biomarkers.

A related study examined a similar panel of biomarkers in 46 foundry workers with B(*a*)P exposures ranging from <5 to 60 ng/m^3 [Perera,

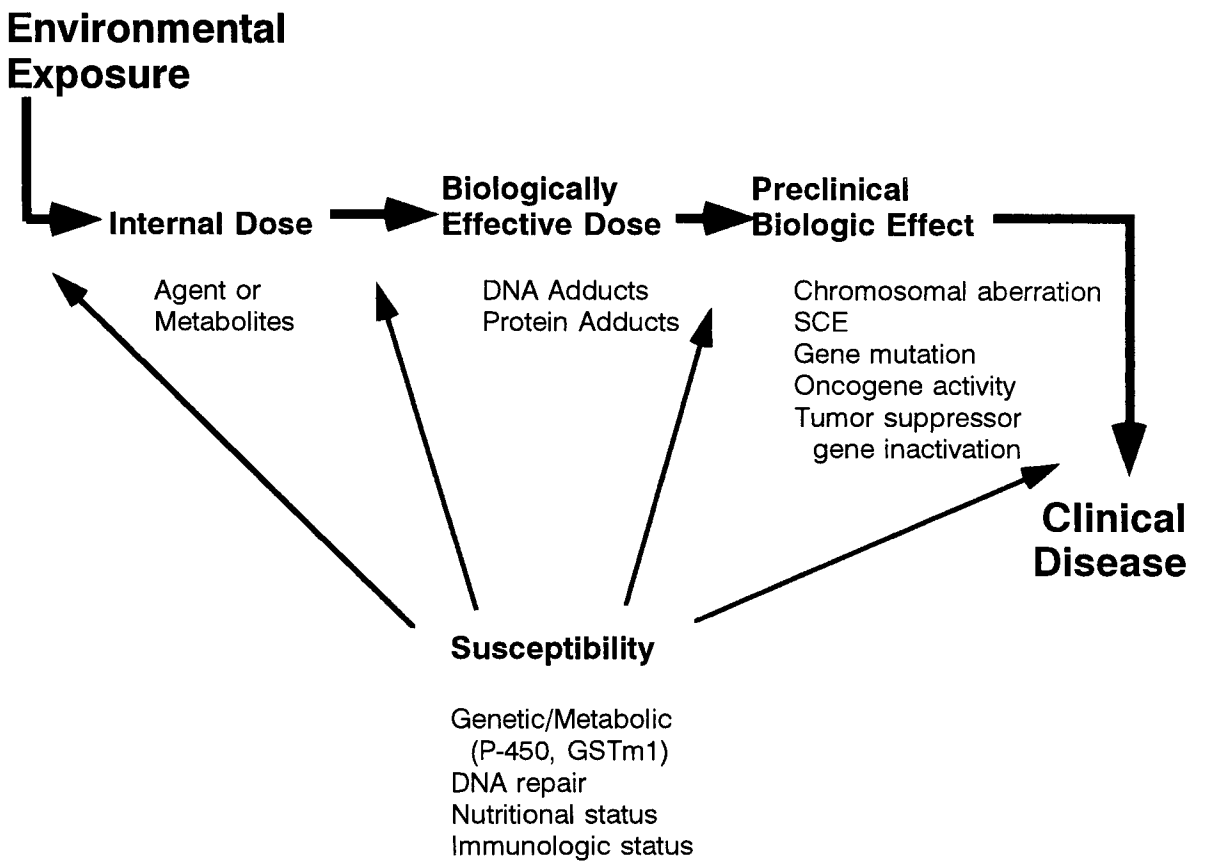

Fig. 1. Categories of biomarkers arranged along the continuum between exposure and clinical disease.

in press]. Of particular interest was the observation that in 17 workers evaluated for adducts and mutations, hypoxanthine guanine phosphoribosyl transferase (HPRT) mutation frequencies were highly correlated with PAH–DNA adducts ($r = 0.67$; $p = 0.004$). This finding of a strong association between a chemical-specific marker of biologic dose and response is consistent with experimental data on mutation induction by PAHs.

In a molecular epidemiologic study of lung cancer cases ($n = 145$ and non-cancer controls $n = 115$), PAH–DNA adducts were measured by enzyme-linked immunosorbent assay (ELISA) in both peripheral blood leukocytes from cases and controls, and lung tumor specimens collected from cases at surgery [Tang, unpublished]. SCEs in lymphocytes, H-*ras* oncogene activation, retinol, and carotenoids were evaluated in serum (see below for discussion). Information on smoking, diet, and occupational exposure was collected. Consistent with a prior study [3], PAH–DNA adducts in leukocytes were significantly higher in cases than in controls after adjusting for number of cigarettes/day. PAH–DNA adducts were also associated with pack-years of smoking. Adducts in peripheral leukocytes showed a significant correlation with adducts in the lung tumor tissue. SCE and H-*ras* overexpression were elevated in cases compared to controls, but the differences were not statistically significant. The association between SCE and pack-years of smoking was significant.

In another study, PAH–DNA adducts were measured by ELISA in lymphocytes from 63 heavy smokers and 27 nonsmokers [4]. Adduct levels and cotinine, a tobacco urinary metabolite, were significantly elevated in smokers compared to controls ($p < 0.001$). However, neither

TABLE I. Biomarkers in Populations With Inhalation Exposure to Environmental Carcinogens

Compound Analyzed	Exposure/Source	Biologic Sample	Population	References
Internal Dose				
CFA	Occupational exposure	Urine	Workers	[47]
1-Hydroxypyrene	Coal tar products	Urine	Workers	[48]
Mutagens	Various occupational exposures	Urine	Workers	[49]
Mandelic Acid	Styrene	Urine	Workers	[50]
Biologically Effective Dose				
Alkylated Hb	Propylene oxide	RBC	Workers	[51]
Hydroxyethylhistidine, hydroxyethylvaline	Ethylene oxide	RBC	Workers	[52] [53] [54] [55] [56]
PAH–Albumin	PAHs in workplace	Plasma	Workers	[57]
PAH–DNA	PAHs	WBC	Workers	[58]
Spectrum of DNA adducts	Various industrial exposures	WBC	Workers	[58]

Early Biologic Effect of Response

Chromosomal aberrations	Industrial chemicals radiation	WBC	Workers	[53] [59] [60] [61]
DNA hyperploidy	Aromatic amines	Bladder and lung cells	Workers	[62]
HPRT mutation	Radiation	WBC	Workers	[63]
Micronuclei	Organic solvents, heavy metals	WBC	Workers	[64]
Oncogene activation	PAHs	Serum	Workers	[65]
Single strand breaks	Styrene	WBC	Workers	[66] [50]
Sister chromatid exchange	Industrial chemical exposure, radiation	WBC	Workers	[67] [68]
Unscheduled DNA synthesis	Propylene oxide	WBC	Workers	[69]

CFA, 3-chloro-4-fluoroaniline
Hb, hemoglobin
RBC, red blood cells
PAHs, polycyclic aromatic hydrocarbons
WBC, white blood cells
HPRT, hypoxanthine guanine phosphoribosyl transferase
PAHs, polycyclic aromatic hydrocarbons
WBC, white blood cells

cotinine nor DNA adducts in smokers correlated with the amount of smoking (cigarettes per day or pack-years). As described below, the role of micronutrients was examined as one possible contributor to the observed inter-individual variability.

Taken in the context of the research summarized in Table I, these results demonstrate the ability of multiple biomarkers to detect genetic and molecular damage in humans over a wide spectrum of exposure. It is also apparent that biomarkers are capable of documenting inter-individual variation and unraveling the factors contributing to this variation. What is less apparent from a general summary of this nature is the complexity of research design and analysis needed to control appropriately for confounders and other variables introduced by this interdisciplinary approach [for review see 5].

EXPERIMENTAL AND EPIDEMIOLOGIC EVIDENCE FOR A CHEMOPREVENTIVE EFFECT OF MICRONUTRIENTS IN LUNG CANCER

The evidence for a protective effect of micronutrients in lung cancer is briefly summarized. The available data largely concern antioxidants and free radical scavengers such as vitamin C, β-carotene (β-C), the carotenoids, vitamin E, and retinol [for review see 6,7].

Vitamin C effectively scavenges oxygen radicals, preventing free radical damage by superoxides and regenerating the tocopheroxyl radical. For example, vitamin C inhibits the metabolic conversion of PAH compounds to their carcinogenic intermediates, blocks nitrosamine formation by reducing nitrite, and has been shown to detoxify various organochlorine pesticides (DDT, dieldrin, lindane, *etc.*) [7]. Vitamin C has been found to affect metabolic activation and detoxification of PAHs by inhibiting cytochrome P-450 [8] and microsomal aryl hydrocarbon hydroxylase (AHH) activity *in vitro* [9]. Shah *et al.* [10] reported decreased *in vitro* binding of B(*a*)P to DNA. Other effects of vitamin C include the reduction of DNA adduct formation in rat lung by cigarette smoke condensate [11] and suppression of mutagenesis in *Salmonella* by aflatoxin B_1 (AFB_1) [12]. In mice, vitamin C inhibits the induction of lung neoplasms by B(*a*)P, nitrogen-containing compounds, fiberglass, and plutonium oxide, and protects against the transformation of hamster lung cultures by cigarette smoke [7,13].

Beta-carotene is an antioxidant, quenches singlet oxygen, and like other carotenoids, affects cellular differentiation and enhances immune function [6]. It also enhances the activity of intestinal AHH in rats [14] and selectively increases the rat liver cytochrome P-450-mediated monoxygenase system for B(*a*)P detoxification [15]. In experimental systems, β-C suppresses the formation of carcinogen–DNA adducts by diverse carcinogens including 7,12-dimethylbenz(*a*)anthracene (DMBA) [16] and AFB_1 [17] as well as the induction of SCE by DMBA, *N*-nitrosodiethylamine (DEN), and *N*-methylnitrosourea (MNU) [18]. Beta-carotene suppresses the mutagenicity of B(*a*)P and other mutagens in *Salmonella* [19,20] as well as cell transformation by diverse chemicals [21]. Experimentally, some but not all studies have reported that β-C inhibits the induction of UV- and chemically induced tumors [6]. In humans, the formation of micronuclei in smokers [22,23], betel quid chewers, and snuff takers was reduced following administration of β-C [22].

Retinol and the retinoids enhance cell differentiation, suppress malignant transformation, and counteract the effect of tumor promoters [6]. Experimentally, they reduce the induction of DNA adducts, DNA damage, mutation, and/or SCE by such diverse carcinogens as B(*a*)P, DMBA, AFB_1, dimethylnitrosamine (DMN), and DEN [24–29]. In human lung cancer cells, retinoids increased the expression of the tumor suppressor gene p53 [30]. There is evidence that vitamin A deficiency strongly increases B(*a*)P mutagenicity [31]. Activity of cytochrome P-450-dependent mixed-function oxidases, the major enzyme system involved in B(*a*)P metabolism and activation, has been found to decrease with vitamin A deficiency [32,33]. Glutathione-*S*-transferase (GST) has also been reported to decrease in various cells and tissues with vitamin A deficiency [34,35].

Vitamin E protects against damage by radicals, reduces oxidative breakdown of fatty acids, scavenges oxygen radicals, and blocks the formation of nitrosamine [for review see 7]. Vitamin E inhibited both the formation of B(*a*)P metabolites and PAH-DNA binding [36]. Vitamin E also decreased mutagenesis in *Salmonel-*

la by B(*a*)P and AFB_1 [12]. Experimentally, low doses of Vitamin E combined with β-C suppressed formation of SCE by methyl-*N*-nitrosoguanine (MNNG) and 4-ABP [37]. Experimental and *in vitro* studies have suggested that vitamin E can enhance GST activity [38] and also increase P-450 levels in certain tissues [39,40]. GST can catalyze the conjugation reaction between glutathione (GSH) and substrates bearing hydrophobic and electrophilic sites [41], thereby enhancing detoxification and elimination of various carcinogens.

Epidemiologic studies show a convincing protective effect of fruits and vegetables rich in vitamin C and β-C for epithelial cancers including the lung [for review see 7]. Twenty-nine of 31 studies reviewed by Block showed a significant effect for lung cancer. Overall for the major epithelial cancers, 120/130 studies showed a statistically significant reduction in risk by vitamins C and E, and β-C, or food sources rich in these micronutrients. By contrast, the epidemiologic evidence for a protective effect of retinol is far less compelling [7,42].

At the present time, approximately 40 clinical trials are currently being sponsored by the NCI, aimed at evaluating the efficacy of β-C, retinol, retinoic acid, vitamins C and D, and several other agents in the prevention of various types of cancer [43].

MOLECULAR EPIDEMIOLOGIC EVIDENCE OF A PROTECTIVE EFFECT OF MICRONUTRIENTS IN LUNG CANCER

Biologic markers already play an important role in evaluating chemopreventive agents, specifically in Phase II trials [43–45]. The most commonly used genetic markers in current trials involving lung and upper aerodigestive tract tumors include micronuclei, DNA content, and genetic alteration in oncogenes, as well as markers of proliferation, growth regulation, and differentiation [44]. Our results suggest that additional biomarkers of genetic damage such as carcinogen–DNA adducts can be useful in intervention studies of exposed or "at risk" populations.

Two of our own recent studies examined whether micronutrient levels of vitamins C and E, or β-C or retinol in the serum of persons who have not been treated with chemopreventive agents, are inversely related to biomarkers that measure genetic damage and were previously associated both with exposure to environmental carcinogens/mutagens and/or with lung cancer risk.

In the lung cancer case-control study described above, the serum level of β-C was evaluated in 145 cases and 115 controls. Preliminary analysis showed that β-C was inversely correlated with PAH–DNA adducts in peripheral leukocytes of cases and controls, respectively, and combined. However, the association was not statistically significant.

In the cross-sectional study of 63 heavy smokers mentioned earlier, serum concentrations of cholesterol-adjusted α-tocopherol, ascorbic acid, β-C, and retinol were evaluated in relationship to the levels of PAH–DNA adducts in lymphocytes measured in the same individuals [46]. All participants were current smokers; ninety percent had smoked one or more packs per day for at least 10 years. A significant inverse correlation was found between serum α-tocopherol and PAH–DNA adducts. Although the relationship was not statistically significant, serum ascorbic acid, β-C, and retinol were inversely correlated with adducts.

RECOMMENDED STRATEGIES FOR FUTURE RESEARCH

Environmentally exposed populations should be studied further to determine the relationship between serum micronutrients and the levels of biomarkers which provide information on the biologically effective dose of environmental carcinogens and the preclinical biologic effects of exposure. In addition, as discussed above, the activity of certain markers of susceptibility, *e.g.*, GST, may be modulated by micronutrients such as α-tocopherol. These markers should also be included to examine *in vivo* interactions.

Case-control studies "nested" within prospective studies provide a valuable opportunity to evaluate these relationships in "baseline" blood samples drawn at the time of subject enrollment into a prospective study of lung cancer. Individuals who subsequently develop the disease are matched to controls, and biomarkers are analyzed in the stored samples. This approach gives information about the predictive value of mark-

ers and the mechanistic interactions between them.

Finally, intervention studies should be carried out in smokers or in workers who were either formerly exposed or are currently exposed to carcinogens without the immediate prospect of cessation. Subjects would be randomly assigned to treatment groups (retinoids, β-C, vitamins E and/or C) and levels of biomarkers monitored in serial samples. The substantial promise of chemoprevention justifies further biomarker research to answer basic questions about mechanisms, dose-response and inter-individual variation in response.

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