Biomarkers in Cancer Chemoprevention
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Biomarkers and surrogacy: relevance to chemoprevention

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Clinical cancer prevention trials that use disease as the end-point are of necessity large, lengthy and costly. While such trials will always remain the 'gold standard' for establishing efficacy, they are unwieldy and inefficient for the rapid translation of our accelerating understanding of the molecular basis of cancer into preventive strategies. The inclusion of biomarkers in the process of chemopreventive agent development is crucial for the advancement of the field. This overview highlights the types of approach that are being used in the development and application of biomarkers in chemoprevention studies. Biomarkers, which measure exposure, susceptibility or risk factors, can be used in selecting study cohorts, assessing participant compliance and/or determining agent efficacy. Key features of biomarkers include reliability, precision, accuracy and validity. Not all biomarkers are suitable for all purposes and are likely to be imperfect in any single setting. Judicious selection and matching of biomarkers with agents and study cohorts is required for their effective utilization. A critical but non-dichotomous element of risk biomarkers is their degree of surrogacy. A classification scheme is provided that relates the degree of surrogacy of risk biomarkers to their utility in preventive interventions.

Introduction

The past decade has witnessed the development of an impressive array of biomarkers reflecting specific exposures to environmental agents and/or predicting disease risk in individuals. A biomarker may be defined as a chemical (or infectious) agent in accessible body matrices, an *in vivo* response to an exposure or set of exposures, or a genotype or phenotype indicative of susceptibility to disease, all measurable in body fluids, cells or tissues. Biomarkers have the potential to make possible better assessment of ambient environmental exposures; better methods for risk estimation and classification of at-risk individuals, communities and populations; better definition of mechanisms of exposure–disease linkages and the underlying susceptibility factors; clearer definition of the interactions of multiple agents and exposures on disease outcomes; and, ultimately, better and faster methods for assessing the effect on disease outcomes of exposure remediation and preventive interventions (Hulka *et al.*, 1990; Schulte & Perera, 1993; Muñoz & Gange, 1998). Many of these goals directly affect the development and maturation of the discipline of cancer chemoprevention.

Clinical cancer prevention trials that use disease as the end-point are of necessity large, lengthy and costly. While such trials will always remain the 'gold standard' for establishing efficacy, they are unwieldy and inefficient for the rapid translation of our accelerating understanding of the molecular basis of cancer into preventive strategies. Thus, inclusion of biomarkers, despite some intrinsic limitations, in the process of chemopreventive agent development and application is of central importance for the advancement of the field. As discussed by Kelloff *et al.* (1996), the major structural triad that needs to be considered in unison for the development of chemopreventive agents is the 'ABC' of chemoprevention: agents, biomarkers, and cohorts. Biomarkers can be used in three distinct but complementary ways. First, biomarkers can be used in defining study populations by classifying individuals at risk among whom putative preventive interventions are to be evaluated. Second, biomarkers can be used to accelerate assessment of the efficacy of preventive interventions, both in terms of identification of active agents in humans and optimization of their use (e.g., dose and schedule). Third, biomarkers can be

used to monitor compliance to the agent that forms the basis of the intervention.

The mere existence of a biomarker does not mean that it will be useful to the field. At present the possibilities for biased use of biomarkers probably outweigh prospects for their informed use. This concern arises simply because few of the biomarkers now being used in either preclinical or clinical settings have undergone anything approaching rigorous validation. Indeed, paradigms for the validation of biomarkers are still evolving (Freedman *et al.*, 1992; Schulte & Perera, 1993; Groopman & Kensler, 1999) and considerable effort will be required for the validation of current and future biomarkers of potential use in chemoprevention studies. This overview seeks to highlight the types of approach that are being used in the development and application of such biomarkers that, in turn, reflect different components of the multistage, multifactorial process of carcinogenesis. Of particular importance is the recognition of the concept that the utility of biomarkers in prevention studies is not dichotomous (i.e., good or bad), but rather continuous, with some markers more informative than others, depending upon how they are used. Figure 1 provides a conceptual basis for the application of biomarkers in preventive interventions.

Criteria for useful biomarkers

Not all biomarkers are suitable for all purposes. Some will be helpful in selecting study cohorts, others will find use in assessing participant compliance, and others can be applied to determining agent efficacy in prevention trials. There are a number of analytical and biological criteria that define the utility of any given biomarker for chemoprevention studies (Schatzkin *et al.*, 1990; Kelloff *et al.*, 2000).

The development of most biomarkers being considered for application to chemoprevention trials is driven by improvements in analytical methods. Our abilities to measure ever-smaller amounts of molecules in a complex biological milieu provide ever-greater insight into the key pathways of the carcinogenic process and the potential modulating effects of chemopreventive agents. These molecules can be environmental

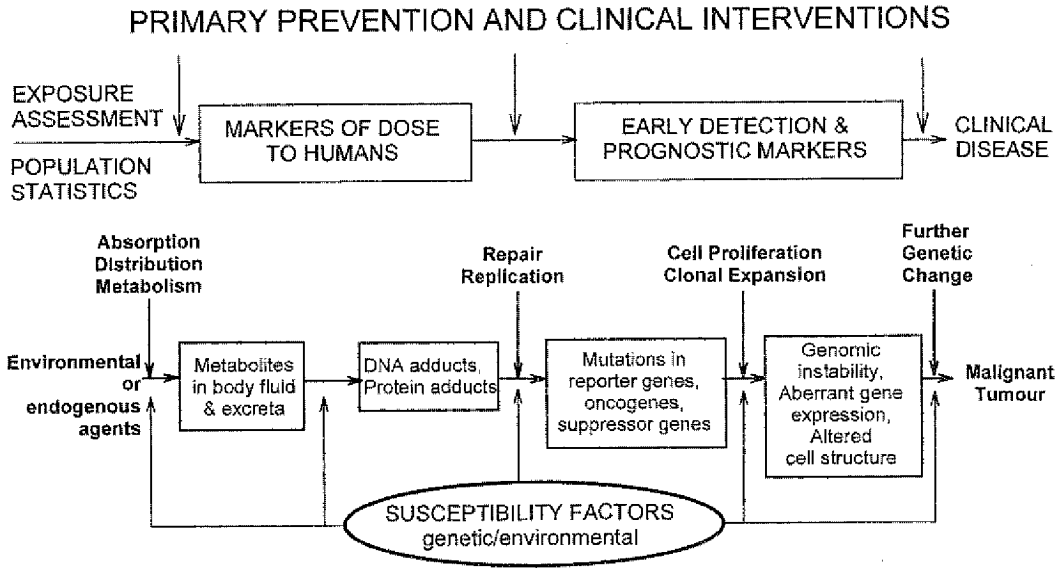

Figure 1. A conceptual basis for the application of biomarkers for use in prevention trials.

carcinogens, oligonucleotide sequences or expressed proteins. Simplicity and cost are important parameters. Complex laboratory-based assays of limited throughput may not be suitable for clinic- or field-based interventions where thousands of samples may be collected for analysis. Moreover, the biomarker needs to be obtained by non-invasive or minimally invasive procedures.

Regardless of intended application, there are fundamental analytical issues that need to be addressed for all biomarker assays. They include reliability, precision, accuracy and validity. Table 1 provides a description of each of these essential characteristics and indicates how they are measured.

Combination of the characteristics defined in Table 1 gives a full description of the properties of a given biomarker. A fully reliable ($S_w^2 = 0$), totally precise ($S^2 = 0$) and accurate ($m - \mu = 0$) biomarker is the desired goal, but this is extremely difficult to attain and almost never happens. It is also unusual to have a fully reliable and totally precise yet inaccurate ($m - \mu \neq 0$) biomarker, but this could be the case with a superb laboratory technique measuring the wrong analyte. From epidemiological and intervention points of view, the ideal biomarker will be fully reliable, reasonably precise ($S^2 > 0$ but not too large), and accurate. In practice, most population-based studies use biomarkers that are moderately reliable ($S^2 > 0$, but not large) and moderately precise ($S^2 > 0$ but not large), and accurate ($m - \mu = 0$).

Most reports document the reliability and precision of the biomarker measurements, but do not directly incorporate them into inferential summaries. Efforts should be made to integrate all aspects of the biomarker in inferences regarding their response to exposures and/or interventions. Another analytical issue that is important is tracking. In the context of a longitudinal study, if exposure is constant over time, the longitudinal measurements of a biomarker could be viewed as the x replicates in the experimental setting outlined in Table 1, so that ρ represents the tracking correlation. The degree of tracking of the biomarker will influence the needed frequency for repetitive sampling.

Of the three characteristics above, accuracy is the primordial one. In general, accuracy imparts validity to the biomarker, but inaccuracy does not preclude validity. As a matter of fact, in the context of chemoprevention trials, where the objective is to quantify the effect of an intervention on disease risk via modulation of one or more biomarkers, it is validity that is the most important feature of a biomarker. In the situation where the biomarker is inaccurate ($m - \mu \neq 0$, that is, biased), the hope is for the biases to operate in the same direction in both control and intervention groups, so that the validity of the study is preserved.

When the primary objective is to use a biomarker as the study end-point to monitor efficacy of the intervention, two biological features are also essential. The first feature is the degree of association between a risk biomarker and disease outcome — cancer. In general, the higher the association, the more useful the biomarker to chemoprevention trials. The second biological feature is that intermediate end-point biomarkers must be modulated by interventions in predictable, dose-dependent ways. These are necessary conditions for a biomarker to be a surrogate marker. Surrogacy is discussed in detail later in this chapter.

Biomarkers as measures of exposure: dose to humans

Humans are exposed to chemical, physical or biological carcinogens through contaminated air, water, soil, food or biological specimens (blood, semen, saliva). Thus, a person's exposure is the result of proximity to the agent superimposed upon many modifying factors. A biomarker of exposure may be the parent chemical itself, as exemplified by heavy metals. Frequently, however, it is a metabolic product of the agent formed in the body that serves as a marker of exposure and provides an internal dose measure. Carcinogen – DNA and carcinogen–protein adducts are also markers of exposure and are often referred to as measures of 'biologically effective' dose. Ideally, biomarkers of exposure should indicate the presence and magnitude of previous exposure to an environmental carcinogen. In the absence of a biomarker, assessment of exposure typically requires measurement of toxicant levels in the environment and characterization of the individual's presence in, and interaction with, that environment. The use of ambient measurements to determine exposure status of individuals is complicated because most etiological agents are not evenly distributed in the

Table 1. Essential characteristics for biomarkers

The experiment used to define measures of reliability, precision, accuracy and validity in this table consists of x replicate (i.e., same conditions) determinations on n homogeneous (i.e., same mean) individuals. The true common mean is denoted by μ; the observed overall mean (mean of individual means) by m; the within-individual error (average of the individual's variances of the sets of m replicates) by S_W^2; the error between individuals (variability of the individual's means around the overall mean) by S_B^2; the total error ($S_W^2 + S_B^2$) by S^2; the correlation between two measurements in an individual (S_B^2/S^2) by ρ; and the bias ($m-\mu$) by b. Measures with an * indicate those of the intervention group. The true difference in means between the intervention and control groups is denoted by Δ.

Concept	Description	Measures	Goals	
Reliability	Repeatability	S_W^2; ρ	$S_W^2 = 0$; $\rho = 1$	Fully reliable
Precision	Sharply measured	$S^2 = S_B^2 + S_W^2$	$S^2 = 0$	Totally precise
Accuracy	Measures 'true' level	$b = m - \mu$	$b = 0$	Unbiased
Validity	Measures 'true' change (or effect of intervention on outcome)	$b^* - b = (m^* - m) - \Delta$	$b^* - b = 0$ or $m^* - m = \Delta$	Internal validity

environment. Thus, the requirements for development of practical biomarkers of exposure must include an ability to integrate various routes and fluctuating exposures over time, to relate time of exposure to dose and to examine mechanisms in important biological targets (Muñoz, 1993). In turn, use of such accurate biomarkers of exposure will limit misclassification, which is often the major source of error in environmental epidemiology (Hulka, 1991). Proper identification of individuals at risk for exposure to cancer-causing agents offers strong potential for enriching the selection of study cohorts (and correspondingly reducing sample size requirements) for chemoprevention trials.

Urinary metabolites

In the early 1800s, Wöhler and his colleagues recognized that urine was a vehicle for the elimination of metabolites of xenobiotics, when they identified hippuric acid following dosing with benzoic acid (Young, 1977). Now, both oxidation and conjugation products of a multitude of drugs and environmental toxicants, including many carcinogens, have been identified and quantified in the urine of humans. Such measurements have become analytical staples for molecular epidemiologists seeking to identify causal linkages between carcinogen exposure and disease (Shuker *et al.*, 1993). Examples of such metabolites include mercapturic acids derived from glutathione conjugation of several carcinogens (De Rooij *et al.*, 1998) including aflatoxin (Wang *et al.*, 1999), benzene (Boogaard & van Sittert, 1995) and 1,3-butadiene (Hayes *et al.*, 2000); glucuronide and sulfate esters of heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Lang *et al.*, 1999); 1-hydroxypyrene in workers exposed to polycyclic aromatic hydrocarbons (Bouchard & Viau, 1999); and oxidation products and glucuronides of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers (Hecht, 1997). These metabolites are obviously strong markers of exposure and in some instances have served as intermediate biomarkers in chemoprevention trials.

Hecht *et al.* (1995) have analysed the effects of consuming watercress, which is a rich source of phenethyl isothiocyanate (PEITC), on the metabolism of tobacco-specific carcinogens in smokers. They observed that watercress consumption increased urinary levels of two metabolites of NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide, NNAL-Gluc. This increase was attributed to either inhibition of cytochrome P450 or induction of glucuronidation. Watercress consumption also affected nicotine metabolism in these individuals, with an elevation in levels of glucuronide of nicotine in urine samples collected during the intervention and levels returning to baseline after the watercress consumption period (Hecht *et al.*, 1999). Likewise, in a large, double-blinded, placebo-controlled trial of oltipraz conducted in the People's Republic of China, urinary markers were used to demonstrate pharmacodynamic action by the intervention agent. Measurements of phase I (aflatoxin M_1) and phase II (aflatoxin-mercapturic acid) metabolites were used to demonstrate that oltipraz inhibited oxidation and enhanced conjugation of aflatoxin relative to placebo (Wang *et al.*, 1999). Reductions in levels of circulating aflatoxin–protein adducts were also seen in participants receiving oltipraz (Kensler *et al.*, 1998).

DNA and protein adducts

While early-stage measurements provide unequivocal identification of chemical exposures, they do not provide evidence that toxicological damage has occurred. Measurements of carcinogen–DNA and carcinogen–protein adducts are of interest because they provide molecular, mechanism-based bridges between carcinogen exposures and disease end-points. These adducts are direct products of damage to critical macromolecular targets and reflect an integration of the toxicokinetic factors of absorption, distribution and metabolism. However, these toxicokinetic factors are not constant and can vary as a function of dose and duration of exposure. Replication of carcinogen-modified DNA is thought to result in the fixation of mutations that serve as initiating agents in transformation and, thus, formation of such adducts is assumed to be on the causal pathway. However, since the pattern and level of these adducts can also be profoundly influenced by repair processes of differing efficiency and fidelity, the usefulness of measurements of DNA adduct concentration to predict cancer incidence quantitatively remains unclear (Gaylor *et al.*, 1992). Indeed, several estimates of the overall contributions of carcinogen adducts to cancer risk, using animal models in which exposure can be carefully controlled, indicate that the attributable risk for these types of marker may be less than 10% (Travis *et al.*, 1996; Kensler *et al.*, 1997).

Given the complex interactive nature of the carcinogenic process, it is unreasonable to expect that a single, early marker can fully predict risk of cancer outcomes. Clearly, production of genetic damage by carcinogens is not a *sine qua non* for cancer. Nonetheless, like the metabolite markers for internal dose, the adduct biomarkers effectively delineate exposures and serve as modulatable endpoints for judging the efficacy of certain classes of chemopreventive agents, notably those that protect cells by altering the metabolism and disposition of the reactive intermediates leading to DNA damage (Kensler, 1997). Indeed, modulation of carcinogen adduct levels has been used for a considerable period of time as a short-term endpoint for the initial evaluation of chemopreventive agents *in vivo* (Kensler *et al.*, 1985). In general, the population-based predictive value is quite good. However, in some instances, especially when complex tumour induction regimens are used involving tumour initiators and promoters, correlations between adduct levels and ultimate tumour yields can be poor, or more perversely, negative (Hartig *et al.*, 2000). In the one case where levels of adducts were assessed for their predictive value of individual risk for developing cancer, no value was observed, despite the fact that a strong correlation was observed in the same experiment between adduct burden and treatment group risks for hepatocarcinogenesis (Kensler *et al.*, 1997). Once the individual animal was identified by treatment group assignment, the adduct biomarker provided no further information about cancer risk.

A variety of analytical methods are now available for detecting and quantifying covalent adducts formed between DNA and proteins and genotoxic chemicals. Methods for DNA adduct analysis include immunoassay, ^{32}P-postlabelling and physicochemical procedures based on such properties as fluorescence or involving mass spectrometry and electrochemical analysis. Protein

adducts in haemoglobin and serum albumin can be analysed by physicochemical methods, principally gas chromatography/mass spectrometry, or by immunoassay. Collectively, methods are now available for the detection of DNA and/or protein adducts of many of the major classes of chemical carcinogens (Kaderlik *et al.*, 1992; Shuker & Farmer, 1992; Strickland *et al.*, 1993). These techniques have been used to measure composite and specific DNA adducts in cellular DNA isolated from peripheral lymphocytes, and from bladder and colonic tissues, as well as DNA adducts excreted in urine. Many of these methods are of sufficient sensitivity and specificity to detect ambient levels of exposure and are being applied to studies of tobacco use (polycyclic aromatic hydrocarbons, aromatic amines, tobacco-specific nitrosamines; dietary exposures (aflatoxins, *N*-nitrosamines, heterocyclic amines); medicinal exposures (cisplatin, alkylating agents, 8-methoxypsoralen, ultraviolet photoproducts); occupational exposures (aromatic amines, polycyclic aromatic hydrocarbons, 1,3-butadiene, oxides of styrene and ethylene, vinyl chloride); and oxidative damage (8-oxoguanine, thymine glycol, malondialdehyde) (Kensler *et al.*, 1996; Halliwell, 1998; Singer & Bartsch, 1999).

There are a few instances where adducts have been used as biomarkers in human intervention studies. Excretion of 8-oxodeoxyguanosine is associated with age, metabolic rate, caloric intake and antioxidant content of the diet (Fraga *et al.*, 1990; Simic, 1994). Simic & Bergtold (1991) investigated the effects of manipulation of the human diet on levels of urinary markers of DNA base damage, namely, thymidine glycol and 8-oxodeoxyguanosine. Excretion of biomarkers of oxidative DNA damage was suppressed when dietary composition was maintained but caloric intake was decreased. At isocaloric dietary intake, the level of damage depended upon diet composition. For diets containing carbohydrates, proteins and fats but lacking fruits and vegetables, the level of damage was higher than for diets including fruits and vegetables, which are rich in natural antioxidants. Feeding Brussels sprouts to healthy, nonsmoking volunteers also led to a small (28%) but statistically significant reduction in urinary excretion of 8-oxodeoxyguanosine (Verhagen *et al.*, 1995). Similarly, consumption of tomatoes was sufficient to alter levels of oxidative DNA base damage in white cells within 24 h (Rehman *et al.*, 1999). Clearly, levels of these biomarkers can be modulated in humans, making them attractive candidates for assessing the efficacy of antioxidant-based chemoprevention interventions. Dyke *et al.* (1994) have examined the effect of oral vitamin C supplementation on gastric mucosal DNA damage, as measured by ^{32}P-postlabelling in 43 patients. Gastric mucosal DNA damage was decreased in 28 of the patients after vitamin C supplementation ($p = 0.01$). Wallin *et al.* (1995) investigated the effect of phenobarbital (a phase I and II enzyme inducer) treatment of epileptic patients on levels of aromatic amine–haemoglobin adducts as a function of tobacco consumption. In comparison with patients receiving other anticonvulsants, a significant depression in adduct biomarker levels was observed with phenobarbital treatment.

Biomarkers as intermediate measures of disease: early detection and prognostic markers

The historical precursors to biomarkers in cancer research arose from the quest to discover cancer at an early and treatable stage. Numerous such 'tumour markers' are used currently to diagnose or confirm diagnosis for specific cancer types. Examples include carcinoembryonic antigen (CEA) for tumours at several sites, prostate-specific antigen (PSA) for carcinoma of the prostate, 5-hydroxy-indoleacetic acid in the urine for carcinoid tumours, α-fetoprotein for liver cancer and germ-cell tumours, and thyrocalcitonin for medullary carcinoma of the thyroid (Keefe & Meyskens, 2000). As reviewed by Schulte & Perera (1993), the history of tumour marker research, particularly in the areas of cancer cytology and cytogenetics, also provides examples of past attempts to validate markers and bring them into screening programmes. The use of Papanicolaou cytology as a marker of preclinical cervical cancer demonstrates how a good marker can lead to effective intervention, yet 27 years lapsed between the development and adoption of the Pap test (Greenwald *et al.*, 1990). In addition to the search for early detection markers, prognostic and predictive markers have been developed. These markers help guide decision-making about therapeutic options and opportunities.

Somatic mutations and genomic instability

Carcinogenesis is driven by an accumulation of genetic changes. These changes occur over time and lead to the evolution of extended clonal foci of neoplastic cells. A variety of detection methods have been developed to detect the presence of neoplastic cells in accessible samples of body fluids and tissues. Mutations are among the earliest lesions to occur following assault of the genome by endogenous or exogenous carcinogens.

Mutations can be detected in easily obtained cell types in reporter genes, whose modification is unrelated to the causal development of cancer, but which reflect exposures to carcinogens. The detection of mutations in the *HPRT* gene is currently the most extensively employed assay for detecting somatic mutations in human genes *in vivo*. *HPRT* mutations are examined in lymphocytes, and the standard assay involves T-lymphocyte cloning for phenotypic selection of 6-thioguanine-resistant mutant cells (Tates *et al.*, 1991). The location and type of mutations in a specific sequence of nucleotides defines the mutational spectrum and have been analysed in the *HPRT* locus of lymphocytes from humans exposed to a variety of genotoxins (Cole & Skopek, 1994). Another *in vivo* assay for the detection of somatic mutations is the glycophorin A assay. This assay is based on the autosomal glycophorin A locus that encodes the cell surface sialoglycoprotein expressed in the erythrocytic lineage and responsible for the M,N blood group (Grant & Bigbee, 1993). Most of the variants are derived from mutations in bone marrow stem cells and are, therefore, permanent, delineating lifetime exposures to mutagens and accumulated mutations. While rapid, facile and inexpensive, the assay is suitable for only one half of the human population, the M/N heterozygotes.

Significant attention has been focused in recent years on target genes for somatic mutations, oncogenes and tumour-suppressor genes. Such genes have been classified as gatekeeper and caretaker genes in terms of their control of net cellular proliferation or maintenance of genomic integrity, respectively (Kinzler & Vogelstein, 1997). The most prominent example of a gatekeeper is the *APC* gene in colorectal cancer. Alterations in *APC* lead to derangements of cellular proliferation pathways and mutation of *APC* is thought to be an early event in the process of colon carcinogenesis. Other gatekeeper genes frequently subject to mutation, such as K-*ras* and *p53*, appear to play important roles in later stages of carcinogenesis. The *p53* gene is well suited for mutational spectrum analyses for several reasons. First, it is commonly mutated in many human cancers. Second, the *p53* gene is small, permitting study of the entire coding region. Third, the point mutations that alter p53 function are distributed over a large region of the molecule, allowing extensive inferences of the mechanism of DNA damage involved (Hussain & Harris, 1998). While tumour-specific *p53* mutations have been identified in several human cancers, identification of individuals harbouring such mutations has been problematic. However, DNA can be isolated from the plasma (or serum) of patients with cancer and this plasma carries the same genetic mutations as DNA in the tumour (Nawroz *et al.*, 1996). Kirk *et al.* (2000) have analysed for a selective arginine-to-serine substitution in codon 249 of *p53* in DNA isolated from plasma. This codon is a hotspot for mutation in hepatocellular carcinoma occurring in populations that are exposed to aflatoxins and have a high prevalence of infection with hepatitis B virus (Hollstein, 1991). The 249-Ser mutation in *p53* was detected in DNA isolated from plasma by restriction endonuclease digestion of polymerase chain reaction products from exon 7 of the gene. Its presence is strongly associated with hepatocellular carcinoma in patients from The Gambia, a high-risk region, but not in patients with liver cancer from Europe. Such approaches allow earlier detection of liver cancer and provide possible intermediate end-points for assessing the impact of intervention programmes such as hepatitis B vaccination and chemoprevention. Mutations in gatekeeper genes can also be assessed in other settings such as *ras* gene mutations in stool (Sidransky *et al.*, 1992) and *p53* mutations in exfoliated bladder epithelial cells isolated from urine (Sidransky *et al.*, 1991). An exciting recent development is the measurement of mitochondrial DNA mutants in tumours and body fluids (Fliss *et al.*, 2000). By virtue of their clonal nature and high copy number, mitochondrial mutations may provide particularly sensitive markers for noninvasive detection of early neoplastic lesions.

It now appears that 4–10 events are necessary for the development of sporadic solid tumours (Fearon & Vogelstein, 1990); however, the normal

baseline rate of mutation within a cell is insufficient to account for the required number of events (Loeb, 1998). Early inactivation of genes that maintain genomic stability (caretaker genes) could result in a mutator phenotype that would significantly destabilize the genome, increase the mutation rate and lead to tumour progression. Such genomic instability, reflecting the propensity and susceptibility of the genome to acquire multiple alterations, is believed to be the driving force behind multistage carcinogenesis. Genomic instability is manifest in several forms: aneuploidy, microsatellite instability and intrachromosomal instability. Probably the best characterized form of instability is microsatellite instability. It involves the insertion or deletion of one or two base pairs in simple repeat sequences (Perucho, 1996) and can result from inherited or somatic defects in DNA mismatch repair genes (e.g., *hMSH2* and *hMLH1*). Diagnostic assays have been developed for microsatellite instability in body fluids. Squamous cell carcinoma of the aerodigestive tract and bladder cancer can be detected through microsatellite analysis of saliva and urine, respectively (Mao *et al.*, 1996; Steiner *et al.*, 1997; Spafford *et al.*, 1998). Renal cancers can also be detected by molecular urinalysis (Eisenberger *et al.*, 1999), while early-stage lung cancer has been detected in tumour DNA isolated through bronchoalveolar lavage (Ahrendt *et al.*, 1999; Field *et al.*, 1999) and sputum (Mao *et al.*, 1994). Moreover, collateral microsatellite analysis of serum samples in some of these studies reveals evidence of circulating tumour DNA and may portend poorer prognosis. Nipple aspirates provide avenues for cytological and molecular analyses in breast cancer prevention trials (Fabian *et al.*, 1997). FISH assays are also available to diagnose and monitor the treatment of field cancerization, i.e., of diffuse genomic instability, even before the onset of intraepithelial neoplasia in patients with proven high risk of cancer (e.g., previous surgery for head and neck cancer). Resection, therapy and/or prophylaxis may all be appropriate in individuals manifesting markers of genomic instability.

Aberrant gene expression

Altered patterns of DNA methylation are among the earliest molecular changes to occur in the evolution of neoplastic cells. In particular, aberrant methylation of CpG dinucleotides that are clustered in the 5′ flanking and first exonic regions of many genes (CpG islands) appear to occur very early in tumour progression for several tumour types and could alter chromatin structure and/or play a role in the loss of tumour-suppressor or differentiation gene functions. Indeed, aberrant hypermethylation of CpG islands has been implicated in the transcriptional inactivation of many genes including those for Rb, p15, p16, p73, VHL, E-cadherin, TIMP3, glutathione *S*-transferase (GST) Pi, MLH1, BRCA1, estrogen receptor α, progesterone receptor, retinoic acid receptor β and androgen receptor. A critical finding is that aberrant promoter methylation is seldom seen in normal tissues except for imprinted genes and genes on the inactive X chromosome. In addition, hypermethylation changes are fairly constant among tumours and occur within the same regions, that is the promoter region of the target genes. Also, these changes can be assessed in a relatively stable molecule, DNA. PCR-based strategies to assess DNA promoter hypermethylation now exist, providing a sensitive method for detection using minute amounts of biological samples. All these features make detection of promoter region hypermethylation an attractive marker for detection of tumour cells (Laird & Jaenisch, 1996).

The potential clinical utility of this approach has been demonstrated by several pilot studies. For example, in non-small-cell lung cancer, hypermethylation of *p16* was detectable in bronchoalveolar lavage samples from patients with lung cancer whose tumours also had methylation of *p16*, but not from those collected from patients whose tumours did not show this change (Ahrendt *et al.*, 1999). Further, evidence of *p16* methylation has been detected in sputum from patients with lung cancer or those at high risk for lung cancer development (Belinsky *et al.*, 1998). A similar analysis of *p16* hypermethylation in patients with hepatocellular carcinoma showed *p16* methylation in 16/22 liver cancers and similar changes were detected in the plasma or serum of 13 of the 16 cases (Wong *et al.*, 1999). These studies have now been extended to include panels of methylated genes. A prototype analysis examined gene methylation patterns in normal lung, lung cancer and serum from non-small-cell lung cancer patients at the time of surgery. Overall, 15 of 22 tumours showed methy-

lation of one or more of four genes in the tumour and the same alteration was found in the serum of 11 of these 15 patients (Esteller *et al.*, 1999). Similar approaches have been successfully applied to the study of patients with head and neck cancer.

Recent technological advances potentially provide powerful tools for direct analysis of the expression of multiple genes simultaneously in normal and abnormal tissues. These include open systems such as SAGE and closed systems such as microarrays and oligonucleotide chips. This field is currently in its infancy and initial studies are focused upon molecular classification of established tumours as a proof of principle. For example, a preliminary application of a microarray strategy to molecular classification of leukaemias has been described (Golub *et al.*, 1999). A long-range goal of this type of approach could be to predict clinical outcome in both the treatment and prevention settings. In the shorter term, microarray analyses can provide mechanistic readouts on the pharmacodynamic actions of chemopreventive agents.

Intraepithelial neoplasia

Intraepithelial neoplasia (IEN) is a precancerous lesion directly on the causal pathway to cancer and has traditionally been detected by histopathological methods. Two basic processes underlie the onset and development of IEN (Boone *et al.*, 1997). The first is genomic instability, the second is development within an epithelium having genomic instability of multicentric neoplastic lesions that independently progress through each of the following processes at an accelerating rate: clonal evolution, hyperproliferation, production of genomic structural variants, and apoptosis. IEN is the most common intermediate end-point currently applied in chemoprevention trials. It is used both in selection of study cohorts and as an end-point to assess efficacy. For the latter purpose, regression and prevention of recurrence of IEN are assessed. A wide range of IEN have been used for cohort selection for chemoprevention trials, including ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) for breast cancer; prostatic intraepithelial neoplasia (PIN); cervical intraepithelial neoplasia (CIN); adenomatous polyps for colon cancer; and dysplastic lesions of the stomach. Detailed discussions of the use of IEN in defining study cohorts and as outcome measures can be found in other chapters in this volume and in several reviews (Boone *et al.*, 1997; Keefe & Meyskens, 2000). Because IEN are at-risk foci of neoplastic cells, they are fertile regions for applications of molecular laboratory medicine that allow measurement of genomic instability and altered gene expression. Highly quantitative methods for assessing altered cell morphometry have also been developed. Quantitative computer-assisted image analysis systems can be used to measure features of nuclear morphometry such as increased size, altered shape, pleomorphism, altered chromatin texture, DNA ploidy and proliferative index, and are beginning to be applied to measure potential effects of antiproliferative agents in chemoprevention trials (Bacus *et al.*, 1999).

Biomarkers as measures of susceptibility

Epidemiological and human genetic studies have identified different types of at-risk individuals within populations (Harris, 1989). These interindividual differences in susceptibility to carcinogenesis or other diseases may be either acquired or inherited. Some individuals have heavy exposure to environmental carcinogens, while others are carriers of cancer-predisposing germline mutations in genes that, because of high penetrance, confer a very high risk for development of cancer (Dove, 2000). There is also another group of predisposing polymorphic, low-penetrance genes that more modestly increase the risk for cancer in individuals exposed to carcinogens. These genes can be involved in carcinogen metabolism, DNA repair, intracellular signalling (receptors) and immunosurveillance. The proportion of cancers attributable to such genetic traits may be high, because the frequency of these risk-modifying alleles is high in the overall population. In addition, there may be strong interactions between low-penetrance genes, that in the aggregate confer considerable risk for an individual to develop cancer (Hussain & Harris, 1998).

Low-penetrance susceptibility genes

Many enzymes are involved in the oxidative metabolism and conjugation of carcinogens in humans. Some of the genes that control expression of these enzymes are polymorphic and are not expressed in significant percentages of a population. Even for genes that are monomorphic, there

can be huge variations in levels of expression and subsequent enzymatic activity. Thus, both intrinsic (e.g., genetic) and extraconstitutional factors (e.g., diet, hormonal status, occupation) can strongly influence the expression of xenobiotic (and endobiotic) metabolizing enzymes. The molecular basis for the genetic factors leading to variations in activity includes: nucleotide variations in the coding region of the gene (altered substrate binding or turnover rates); deletions in the coding region (inactive enzyme); polymorphisms in the regulatory regions of genes (altered basal or inducible expression); variations in polyadenylation signals (altered transcript half-life and enzyme levels); and gene amplification (increased enzyme levels) (Bartsch *et al.*, 2000). The difficult task lies in identifying individuals who harbour altered capacities for carcinogen metabolism evoked by these mechanisms. Function-based assays such as phenotyping by metabolite analyses of endogenous or exogenous substrates can be informative, but often are analytically laborious. Moreover, metabolic phenotyping is easily affected by confounders such as food or drug intake before testing, which do not affect genotyping assays (Barrett *et al.*, 1997). High-throughput gene analysis by DNA microarray techniques offers prospects for rapid identification of new mutations, while polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods provide easy approaches for characterization of known polymorphisms. However, in these instances, the analytical ease of measurement often outstrips the ability to appreciate the functional significance of these gene variants in humans. Measurements in the absence of understanding of the contributions of specific genetic variations to susceptibility modification are not likely to lead to improvements in the design, conduct and interpretation of chemopreventive interventions.

Genetic polymorphism is probably the single most important determinant of enzyme multiplicity in man and considerable inter-individual variation in drug oxidation and conjugation has been long recognized. Polymorphism refers to a monogenic variation that occurs with at least two phenotypes with sufficient frequency (>1%) to cause population differences. Polymorphisms in many, but not all, phase I (cytochrome P450 (CYP)) and phase II (conjugating) enzymes have been described. Variations in some *CYP* genes have been associated with increased risk for cancers of the lung, oesophagus, and head and neck in smokers (Bartsch *et al.*, 2000; Nair & Bartsch, this volume). Polymorphisms in other *CYP* genes elevate risk for breast cancer, presumably through effects on estrogen metabolism (Feigelson *et al.*, 1998). Polymorphisms in phase II enzymes can also influence cancer risk. In some instances, these enzymes contribute to the metabolic activation of procarcinogens, while in others, their role is in detoxication of reactive intermediates. As examples of this latter case, risk for smoking-related cancers can increase in individuals deficient in GSTM1 (Houlston, 1999; Bartsch *et al.*, 2000). In a more complicated scenario, polymorphisms in *N*-acetyltransferase appear to do both. The rapid acetylator phenotype for acetylation of aromatic and heterocyclic amine carcinogens is associated with increased risk for colon cancer and the slow-metabolizer phenotype with increased risk for urinary bladder cancer (Lang, 1997). Thus, the use of this biomarker as a predictor of individual risk will be dependent upon the context for its use. Moreover, gene–gene interactions between polymorphic phase I and II genes have been observed (Bartsch *et al.*, 2000). Fittingly, the manifestation of the contributions of these susceptibility genes is driven by levels of exposure to carcinogenic substances (Hietanen *et al.*, 1997). Thus, gene-$(n)_x$–gene–environment interactions are the true mediators of risk modification. Metabolic susceptibility genes in the absence of exposure are of little consequence. Development of study cohorts for chemoprevention in this context requires monitoring of biomarkers for both susceptibility and exposure.

DNA repair capacity represents another important susceptibility factor. Patients with the rare, cancer-prone inherited disorder xeroderma pigmentosum experience a greater than 1000-fold excess frequency of sunlight-related skin cancers (Kraemer & Slor, 1985). Laboratory studies indicate that cells from such patients are defective in repairing DNA damage induced by ultraviolet radiation and chemical carcinogens. Although less pronounced, variations in DNA repair capacity have been observed in the general population and may be important susceptibility determinants (Grossman & Wei, 1994). Several assays, notably host-cell reactivation for measuring cellular DNA

repair capacity and an *in vitro* mutagen sensitivity assay, have been developed for application in population-based studies. Correlations between these assays have also been established (Wei *et al.*, 1996). Case–control studies indicate that diminished DNA repair capacity is a risk factor for upper aerodigestive-tract cancers, including lung cancer (Spitz *et al.*, 1996) and basal cell carcinoma of the skin (Grossman, 1997).

In recent years, several genes involved in the repair of mispaired nucleotides (mismatch repair) have been characterized (Bronner *et al.*, 1994). Mutations in these genes are particularly linked to an elevated risk of colon cancer. The mutations occur as heterozygotes and tumours are induced as a result of the loss of wild-type allele. It has been estimated that such mutations are carried by 1 in 200 people, thus constituting one of the most prevalent human disorder mutations (Barrett *et al.*, 1997). While mutations in DNA repair genes can result in loss of DNA repair protein, DNA polymorphisms may alter the structure of the DNA repair enzyme and modulate catalytic activity and efficiency. A recent study evaluated the effects of polymorphisms in the repair enzyme *XRCC1* (X-ray repair cross-complementing 1) in two populations by measuring levels of aflatoxin B_1–DNA adducts in placenta of Taiwanese maternity subjects and somatic glycophorin A variants in erythrocytes from smokers and nonsmokers. In both groups, a Arg399Gln amino acid change appeared to alter the phenotype of the protein, resulting in lowered DNA repair capacity (Lunn *et al.*, 1999).

High-penetrance susceptibility factors

Penetrance is 100% when every individual who carries the mutated gene develops the disease. In the most pronounced cases of familial cancer such as retinoblastoma, affected individuals transmit cancer predisposition to approximately 50% of their offspring (Dove, 2000). In these situations, one mutant allele at a single locus is sufficient for predisposition of individuals for cancer. The set of fully penetrant, dominantly transmitted familial cancers is expanding rapidly. In many cases such familial syndromes yield neoplasms of distinct histological origin and reflect loss of function in the mutated gene.

DNA repair genes provide several examples of loss-of-function familial cancer syndromes. Ataxia telangiectasia, Bloom's syndrome, xeroderma pigmentosum and Fanconi's anaemia lead to dramatically increased risk for lymphoma, solid tumours, skin cancer and acute myelogenous leukaemia, respectively. The aforementioned mismatch repair defects contribute to colorectal, endometrial and gastric carcinoma in patients with hereditary nonpolyposis colorectal cancer (HNPCC). Only a small proportion of colon tumours can be ascribed to members of high-risk families. However, the same gene in which germline mutations are found in high-risk families is often found to be mutated somatically in sporadic tumours at that site. For example, the adenomatous polyposis coli gene, *APC*, is mutated in the germline of familial adenomatous polyposis (FAP), or somatically in HNPCC, and in sporadic colon cancer. Mutations in other tumour-suppressor genes can be observed in multiple types of human tumours and are linked to familial syndromes (e.g., *p53* in Li–Fraumeni syndrome; *BRCA1* and *BRCA2* in familial breast and ovarian cancer).

Some of these genetic syndromes may represent suitable cohorts for inclusion in chemoprevention trials, although for the rarer forms, the need to accrue sufficient numbers of participants into trials may limit this approach. Although FAP comprises only 1% of colon cancer patients, there are some notable examples of chemoprevention trials in this cohort. FAP patients tend to develop thousands of adenomatous polyps, which are evenly distributed throughout the colon and rectum by the third decade of life. In the absence of surgical treatment, affected individuals are at high risk for development of colon cancer by the age of 40 years (Erbe, 1976). Numerous trials have been conducted in patients with polyps to prevent polyp recurrence using pharmacological (sulindac, celecoxib, aspirin, difluoromethylornithine, butyrate) as well as nutritional (fibre, calcium) approaches. These interventions have targeted the proliferative cancer phenotype of the polyps, rather than the underlying predisposing genetic defects. Use of a pharmacological agent to replace the function of a lost or mutated allele is beyond the bounds of current chemoprophylaxis, but within the promise of molecular medicine.

Interactions of susceptibility factors with interventions
Presence or expression of some of the low-penetrance susceptibility genes not only affects risk of carcinogenesis following exposure to genotoxins, but also can modify the potential efficacy of chemopreventive interventions. Many chemopreventive agents undergo metabolism, that may either activate or inactivate the agent. An interesting example of a gene–intervention interaction comes from the major chemopreventive components of cruciferous vegetables, isothiocyanates. These are potent anticarcinogens that act, in part, to induce levels of expression of conjugating enzymes, thereby detoxifying electrophilic forms of carcinogens. Many isothiocyanates are conjugated by GSTs, which facilitate their accumulation in cells (Zhang & Talalay, 1998), but which may also impede the manifestation of their pharmacodynamic actions as enzyme inducers. A case–control study by Lin *et al.* (1998) indicated that intake of broccoli (a cruciferous vegetable) was positively associated with reduced risk for colorectal adenomas. However, when stratified by *GSTM1* genotype, a significant protective effect of broccoli was observed only in subjects with the *GSTM1*-null genotype. In a similar vein, analysis of the interaction between dietary isothiocyanates (measured in urine) and genetic polymorphisms for GSTs in a prospective study conducted in Shanghai indicated that isothiocyanates seemed to decrease lung cancer risk, particularly among persons genetically deficient in the GST isoforms GSTM1 and GSTT1 that may inactivate these chemopreventive compounds (London *et al.*, 2000). We have observed in the chemoprevention trial of another inducer of carcinogen detoxifying enzymes (oltipraz) that the pharmacodynamic action was greatest in individuals who were *GSTM1*-null. In this instance, GSTM1, which is poorly induced by oltipraz, is thought to be the primary constitutive catalyst for the conjugation of aflatoxin with glutathione and likely masks induction of other isoforms of GST. However, in *GSTM1*-null individuals receiving oltipraz, the apparent induction of GSTA1 was unmasked and led to increased excretion of aflatoxin-mercapturic acid (Wang *et al.*, 1999). Clearly, the influence of pharmacogenetics on the actions of chemopreventive agents needs full consideration, as in other aspects of pharmacology such as chemotherapy (Balis & Adamson, 1999).

Degree of surrogacy: a classification paradigm for risk biomarkers

The first step towards the identification of a good early detection/prognostic or 'risk' biomarker is to document the prognostic value of the biomarker (B) on occurrence or incidence of disease (D). Hereafter, we denote this relationship by B→D. The data to document this relationship often come from observational (cohort) studies which describe that natural history of the disease in humans (e.g., cholesterol for cardiovascular disease (Dawber, 1980); human immunodeficiency virus (HIV) load for AIDS (Mellors *et al.*, 1997); persistence of human papillomavirus (HPV) infection for cervical cancer (Ahdieh *et al.*, 2000)) and/or from disease models in animals. The relationship B→D is a prerequisite for a biomarker to have utility in the evaluation of efficacy of a chemoprevention agent (A). Since the primary objective in short-term trials is to use modulation of biomarkers as a measure of efficacy, it is also assumed that evidence is available documenting that the agent modifies the biomarker (i.e., A→B). Such demonstrations often come from animal models.

Under the premises that B→D and A→B, if the agent were not to have an effect on disease (A↛D), this will indicate that the biomarker is useless as an evaluator of the lack of effect of the agent on disease. Furthermore, an effect on this type of biomarker can actually be misleading, as it may suggest a spurious efficacy of the agent. Therefore, a third criterion is that the agent must have an effect on disease occurrence (i.e., A→D). While this criterion is often assumed, establishing this relationship through experimental or clinical studies is in fact very difficult.

Under the abovementioned criteria, a key characteristic of a biomarker is the determination of how much of the effect that an intervention has on disease is captured by the modification of the biomarker during the intervention. This capturing of information is the essence of surrogacy. In the best case, modulation of the marker by the intervention fully captures its effect on disease outcome (full surrogacy). In other words, once the modified (by the intervention) level of the biomarker is determined, no additional information on the

intervention is needed to determine the risk of disease. An important feature of these criteria is to safeguard against using biomarkers that are modified by interventions but have no predictive value for effects on disease onset.

In the 1980s, Prentice (1989) suggested criteria to characterize this full surrogacy, such that, given (or, in statistical terms, conditional on) the biomarker level, there is no residual association between the agent and the disease (A⇏D|B). Unfortunately, there are not many biomarkers that fulfil these stringent criteria. In part, this is because often there are other pathways by which an intervention affects disease that lie beyond the effect on a biomarker (Schatzkin *et al.*, 1990). Many potentially useful biomarkers do not lie directly and exclusively on the causal pathway(s) to disease. A less stringent classification providing a flexible scheme is obtained by quantifying the degree of surrogacy of a biomarker on a continuous rather than dichotomous scale. In terms of simple statistical models, the key comparison is to estimate the relative predictive value of the agent on disease when the biomarker is included as another predictor. Specifically, if the model to relate agent to disease is:

$$\text{Disease} = \alpha + \beta \bullet \text{Agent}$$

where β quantifies the change in disease due to changes in the agent (i.e., β measures A→D), the degree of surrogacy can be determined by comparing β to β^* in the model

$$\text{Disease} = \alpha^* + \beta^* \bullet \text{Agent} + \gamma^* \bullet \text{Biomarker}.$$

Here, the coefficient β^* quantifies the effect of the agent after controlling for the level of the biomarker that, in itself, is modified by the agent. Freedman *et al.* (1992) proposed the use of $(\beta-\beta^*)/\beta$ as a measure of the proportion of the effect of an agent on disease explained by the biomarker. In the more stringent case of the biomarker fully capturing the effect of the agent on disease, one would expect the proportion explained to be equal to 1 (i.e., $\beta^* = 0$). The proportion explained is a direct measure of the degree of surrogacy. The further the proportion explained is from zero, the stronger the degree of surrogacy. As was indicated above, it is very rare to have the proportion explained equal to 1. In turn, the investigator could determine whether a 95% confidence interval for $(\beta-\beta^*)/\beta$ contains one. If so, this result would indicate an ideal degree of surrogacy. Unfortunately, the standard errors for the ratio $(\beta-\beta^*)/\beta$ are typically very large and the inclusion of one by the confidence interval is more likely to be due to the lack of precision in this estimate. The compensatory safeguard is to increase the sample size of the study to a level that defeats the advantages of biomarker studies. Another drawback of estimating the proportion explained is that it is not restricted to be always between zero and one. Biomarker modulation could be bidirectional. Furthermore, the possible residual effects of the agent on disease (after controlling for the attained biomarker levels) may vary by biomarker levels (i.e., interactions between biomarker and agent).

Alternative measures of the degree of surrogacy include the ratio of the measures of the effect of the agent on disease and the effect of the agent on the biomarker (A→D)/(A→B) (Buyse & Molenberghs, 1998). A more epidemiologically based measure would be to require that the change in B due to A is of a magnitude that will correspond to a change in incidence of disease with a strength of a relative incidence below a prespecified level (e.g., 0.80). In other words, if B(A) is the level of the biomarker under the effect of the agent and B(not A) is the level of the biomarker for the control group, one would require that the protective effect of A on D be of a magnitude such that D[B(A)]/D[B(not A)] is less than 0.8. In this case, the reduction of the biomarker by the agent will translate in a reduction of more than 20% of disease occurrence. The more extreme this threshold, the more room there is for an outcome in which, even if not all the change induced by the agent on B translates into change on D, it is likely that the modulation of the biomarker does capture a beneficial effect of the agent.

In the context of HIV epidemiology, the level of HIV RNA in copies/ml provides an example of a biomarker with such a strong prognostic value on AIDS that an intervention modifying HIV viral level was correctly predicted to have an impact on AIDS. Cohort studies documented the strong association between HIV RNA and AIDS (i.e., B→ D), so that the relative hazards for AIDS were 1, 2.4, 4.3,

7.5, and 12.8 for HIV RNA <500, 500–3000, 3000–10 000, 10 000–30 000 and >30 000 copies/ml, respectively (Mellors *et al.*, 1997). In parallel, clinical trials showed that the use of a protease inhibitor-containing combination therapy dramatically reduced the levels of HIV RNA to undetectable levels (A→ B) in a large proportion (~2/3) of individuals (Hammer *et al.*, 1997). The expectation that modulation of HIV RNA was a good surrogate for the effect of protease inhibitor containing combination therapy against AIDS has been realized, and, indeed, therapies have been approved and recommended using HIV RNA as the end-point in clinical trials. After the introduction of these therapies in HIV-infected individuals, cohort studies have shown their effectiveness at the individual level (Philips *et al.*, 1999) and at the population level (Detels *et al.*, 1998; Muñoz *et al.*, 2000).

Classification paradigm for risk biomarkers

The preceding section has described methods to quantify the degree of surrogacy under the assumptions that B→D, A→B and A→D. These three conditions have been referred to as marginal and at-group-level relationships in the statistical and epidemiological literature, respectively. While these three marginal (at-group-level) relationships are necessary, their sufficiency for a biomarker to be a proper evaluator of chemopreventive strategies heavily depends on the degree of surrogacy.

To provide a classification paradigm of risk biomarkers, it is useful to quantify the conditional (at-individual-level) relationships. Specifically, measures should be provided of the conditional relationship of A to D given B ((A→D)|B) and of the conditional relationship of B to D given A ((B→D)|A). In other words, after knowing the attained value of the biomarker, to what extent does one need to also know the intervention assignment to appropriately describe those who developed disease?; and conversely, after knowing whether individuals were treated or not, does one need to also know the biomarker level to characterize disease incidence? Table 2 provides a classification of risk biomarkers according to the existence of these conditional relationships. The Type I biomarkers are those that have prognostic information for disease, that are modulated by the agent and for which the effect of the agent on disease is present at all levels of the biomarker, but, conditional on the agent, the biomarker levels do not predict disease. This type of biomarker, although useful for group comparisons and thus of some utility for evaluation of chemoprevention trials, does not provide information at the individual level about risk modification by the biomarker. The Type II biomarkers are those for which both conditional relationships are present. Namely, the agent modifies disease at all levels of the biomarker and the biomarker predicts disease among those receiving the agent as well as those not receiving the agent. This is likely to be the case for the major-

Table 2. Classification of biomarkers with at-group relationships according to the at-individual relationships

Type	Conditional/individual (A→D)|B	(B→D)|A	β*	γ*	Utility
I	Yes	No	≈β ≠0	≈0	Appropriate for group, but not for individual comparisons
II	Yes	Yes	≠0	≠0	Moderately useful for group and individual comparisons
III	No	Yes	≈0	≠0	Full surrogacy; ideal situation

ity of the biomarkers fulfilling the three necessary marginal (at-group-level) relationships. Biomarkers in this class have predictive value for at-group and at-individual levels and interventions influence disease both through the biomarker and through other means. The Type III biomarker corresponds to full surrogacy, whereby conditional on the biomarker, the agent has no residual effect on disease. In this case, the conditional relationship of the biomarker with disease, given the agent, equates to the marginal prognostic values on disease.

Biomarkers of the Type III or II categories will almost certainly derive from late events in the progression models of human carcinogenesis. High-penetrance genetic susceptibility syndromes and some forms of IEN are good candidates. (The example given above regarding HIV viral load and AIDS is certainly applicable here as well.) However, these markers by no means define the full extent of the population that will actually develop cancer. Additional biomarkers will need to be identified, developed and validated to capture the residual, seemingly low-risk individuals who still develop cancer in the absence of chemoprevention. Low-penetrance susceptibility genes, biomarkers of dose of environmental agents to humans, and some of the newer markers for genomic instability and altered gene expression are potential candidates in this setting. However, many of these biomarkers are likely to have characteristics of Type I, rendering their utility imperfect.

The limitations of Type I biomarkers are briefly highlighted by a study of the value of aflatoxin–albumin adducts for predicting the chemopreventive efficacy of oltipraz against hepatocellular carcinoma in an animal model. Studies in animals and humans have established serum aflatoxin–albumin adducts as biomarkers of exposure to aflatoxin B_1, a food-borne hepatocarcinogen (Wild *et al.*, 1990). To assess the utility of measurements of aflatoxin–albumin adducts in assessing the efficacy of oltipraz for prevention of hepatocellular carcinoma, 82 male F344 rats were dosed with 20 μg aflatoxin B_1 daily for five weeks after randomization into groups given no intervention or intervention (500 ppm oltipraz, during weeks −1 to 5 relative to aflatoxin B_1) (Kensler *et al.*, 1997). In this context, A is oltipraz, B is aflatoxin–albumin adducts and D is hepatocellular carcinoma. Serial blood samples were collected from each animal at weekly intervals throughout the period of aflatoxin B_1 exposure and were assayed for levels of aflatoxin–albumin by radioimmune assay. As shown in Figure 2 (panel a), the area under the curve (AUC) values for overall burden of aflatoxin–albumin adducts decreased by 39% in the oltipraz intervention group compared with no intervention (i.e., A→B). Similarly, total incidence of liver cancer dropped from 83% to 48% ($p < 0.01$) in these groups (i.e., A→D) (panel b). Overall, as shown in Figure 2 (panel c), a significant association ($p = 0.01$) was seen between biomarker AUC and risk of hepatocellular carcinoma (i.e., B→D). However, as shown in Figure 2 (panel d), when the predictive value of aflatoxin–albumin adducts was assessed within treatment groups, there was no association ($p = 0.56$) between AUC and risk of hepatocellular carcinoma (i.e., (B↛D)|A but the association of A to D remained in categories of the biomarker level (A→D)|B). In this case, once the intervention assignment was known, knowledge of the modulated biomarker level provided no further significant information regarding the likelihood of developing cancer for each individual animal. Thus, aflatoxin–albumin adducts can be useful in identifying potential study populations and for monitoring population-based changes induced by interventions, such as in chemoprevention trials, but have, in oltipraz-treated populations, very limited utility in identifying individuals destined to develop hepatocellular carcinoma. Figure 3 graphically depicts the general loci of biomarker types according to the at-individual relationships starting from the at-group relationships and highlights the positioning of the aflatoxin–albumin adduct biomarker as a Type I biomarker.

Conclusions

We have outlined and discussed the properties of biomarkers and challenges faced for their use in evaluating the putative effects of chemopreventive agents on disease and health improvement. These challenges require carefully conducted studies on animals under controlled conditions and studies in humans in which comprehensive data on agents, biomarkers and disease are collected. On top of these challenges, there is the almost universal situation where the determinants of a disease, and therefore, the means for preventing it, are

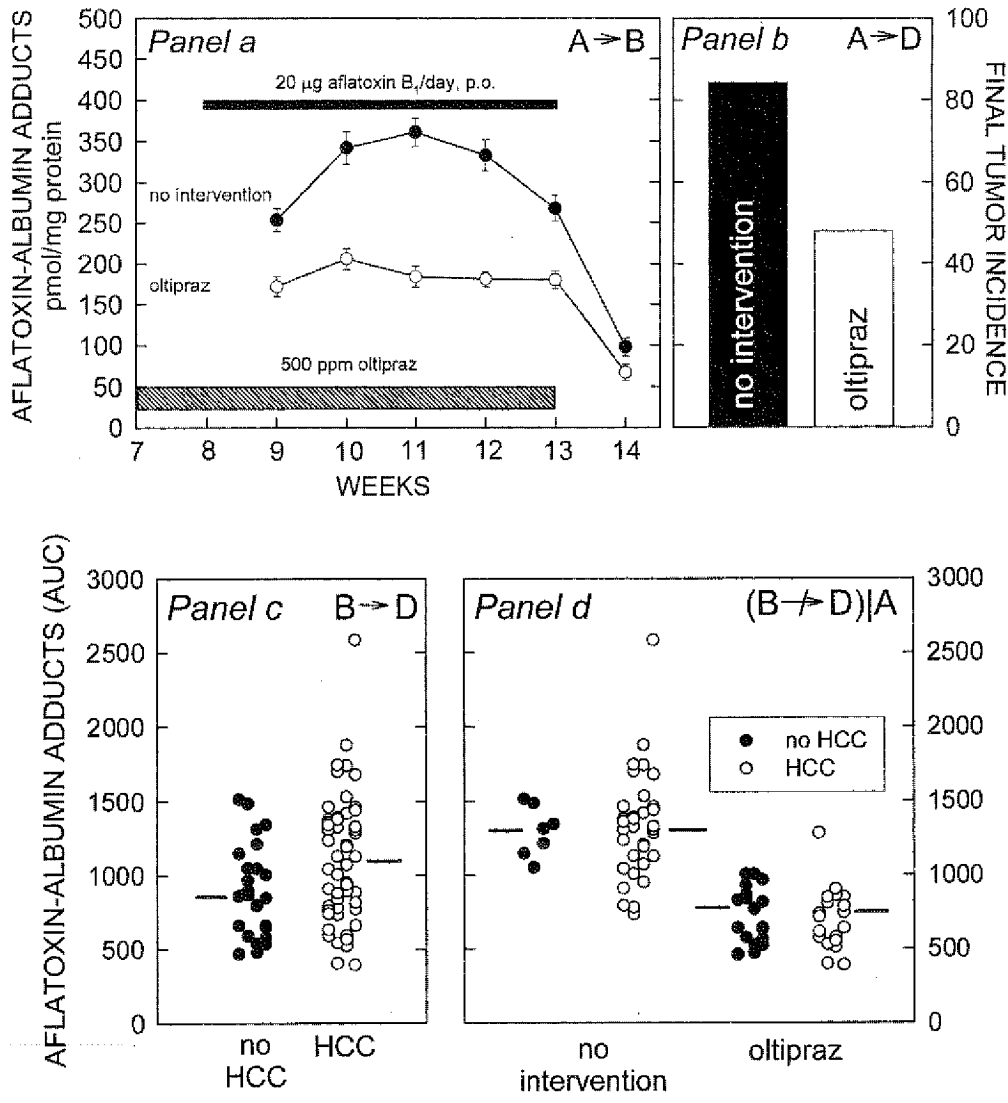

Figure 2. Individual versus group effects of oltipraz on aflatoxin–albumin adduct biomarkers and risk of hepatocellular carcinoma (HCC) in rats.

Panel a, mean serum levels of serial aflatoxin–albumin adducts in rats receiving no intervention or 500 ppm dietary oltipraz. The solid black bar indicates the period of aflatoxin exposure, whereas the striped bar displays the period of oltipraz administration. *Panel b*, effect of oltipraz intervention on incidence of hepatocellular carcinoma (HCC). *Panel c*, univariate association of biomarker burden (AUC: area under curve) with HCC. Biomarker burden was significantly lower in animals that did not develop HCC ($p < 0.01$). Bars, the median of the respective distributions. *Panel d*, bivariate association of AUC with HCC and intervention group.

Adapted from Kensler *et al.*, 1997 with permission.

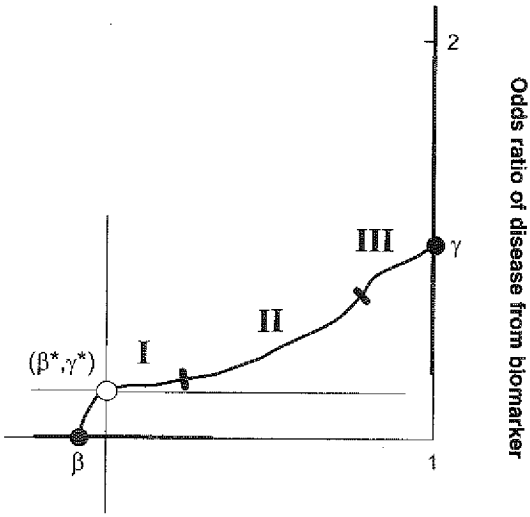

Figure 3. Theoretical distribution of biomarker types for a single agent–disease interaction.

The biomarker occupies one point on this curve. The abscissa corresponds to the value of β^* and the ordinate to the value of γ^*. The intercept on the abscissa corresponds to the value β capturing the at-group relationship of A→D. Similarly, the intercept of the ordinate corresponds to the value γ capturing the at-group relationship of B→D. β and γ can be thought of as measures of the protection conveyed by the (A)gent unadjusted by the (B)iomarker and of the risk of (D)isease predicted by B unadjusted by A, respectively. β^* and γ^* are the corresponding measures following adjustment for B and A, respectively. Values for β and γ and for β^*, γ^* are derived from the data-set depicted in Figure 2. Lines indicate 95% confidence intervals. From the perspective of statistical significance, for Type I biomarkers the values of β^* are significant while those of γ^* are not. Conversely, for Type III biomarkers, the values of γ^* are significant while those of β^* are not. The middle group, where both β^* and γ^* may not be significant, reflects Type II biomarkers.

multifactorial. It is unrealistic to expect that an agent affecting only a specific biomarker will have a major impact in terms of disease prevention. Agents, singly or in combination, that affect multiple components in the process of carcinogenesis are better poised to be effective. Failures of chemopreventive agents and apparent discrepancies between clinical trials and observational studies have more often been due to a lack of a multifactorial approach than to limitations of specific biomarkers or lack of randomization in observational studies. Judicious selection and matching of agents, biomarkers and study cohorts is required. Use of multiple biomarkers that incrementally and collectively enhance surrogacy will be important. In this way, individual biomarkers do not have to be perfect to be useful in advancing the development and evaluation of chemopreventive strategies.

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