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ARTICLES

Risk Biomarkers and Current Strategies for Cancer Chemoprevention

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Abstract Quantifiable, well-characterized cancer risk factors demonstrate the need for chemoprevention and define cohorts for chemopreventive intervention. For chemoprevention, the important cancer risk factors are those that can be measured quantitatively in the subject at risk. These factors, called risk biomarkers, can be used to identify cohorts for chemoprevention. Those modulated by chemopreventive agents may also be used as endpoints in chemoprevention studies. Generally, the risk biomarkers fit into categories based on those previously defined by Hulka: 1) carcinogen exposure, 2) carcinogen exposure/effect, 3) genetic predisposition, 4) intermediate biomarkers of cancer, and 5) previous cancers.

Besides their use in characterizing cohorts for chemoprevention trials, some risk biomarkers can be modulated by chemopreventive agents. These biomarkers may be suitable surrogate endpoints for cancer incidence in chemoprevention intervention trials. The criteria for risk biomarkers defining cohorts and serving as endpoints are the same, except that those defining cohorts are not necessarily modulated by chemopreventive agents. A primary criterion is that the biomarkers fit expected biological mechanisms of early carcinogenesis—i.e., differential expression in normal and high-risk tissue, on or closely linked to the causal pathway for the cancer, and short latency compared with cancer. They must occur in sufficient number to allow their biological and statistical evaluation. Further, the biomarkers should be assayed reliably and quantitatively, measured easily, and correlated to cancer incidence. Particularly important for cancer risk screening in normal subjects is the ability to use noninvasive techniques that are highly specific, sensitive, and quantitative.

Since carcinogenesis is a multipath process, single biomarkers are difficult to correlate to cancer, as they may appear on only one or a few of the many possible causal pathways. As shown in colorectal carcinogenesis, the risks associated with the presence of biomarkers may be additive or synergistic. That is, the accumulation of genetic lesions is the more important determinant of colorectal cancer compared with the presence of any single lesion. Thus, batteries of biomarker abnormalities, particularly those representing the range of carcinogenesis pathways, may prove more useful than single biomarkers both in characterizing cohorts at risk and defining modulatable risks.

Risk biomarkers are already being integrated into many chemoprevention intervention trials. One example is the phase II trial of oltipraz inhibition of carcinogen-DNA adducts in a Chinese population exposed to aflatoxin B_1. Also, urine samples from subjects in this trial will be screened for the effect of oltipraz on urinary mutagens. A second example is a chemoprevention protocol developed for patients at high risk for breast cancer; the cohort is defined both by hereditary risk and the presence of biomarker abnormalities. Modulation of the biomarker abnormalities is a proposed endpoint. Also, dysplastic lesions, such as prostatic intraepithelial neoplasia, oral leukoplakia and colorectal adenomas, have been used to define high-risk cohorts and as potential modulatable surrogate endpoints in chemoprevention trials. J. Cell. Biochem. 25S:1–14. © 1997 Wiley-Liss, Inc.†

Key words: chemoprevention; genetic/regulatory biomarkers; high-risk cohorts; intraepithelial neoplasia; phase II clinical trials, risk biomarkers

Chemoprevention is the inhibition or reversal of carcinogenesis (before malignancy) by intervention with chemical agents. Many epidemiological studies have identified and characterized cancer risk factors [e.g., reviewed in these proceedings by Henderson and Mooney, see also

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1–5]. It may be argued that well-characterized, quantifiable cancer risk factors demonstrate the need for chemoprevention and define cohorts for chemopreventive intervention.

A primary objective of current chemoprevention research is development of strategies for evaluating chemopreventive efficacy in phase II and small phase III clinical trials [6–8]. The discussion following considers the current and potential impact of cancer risk on these chemoprevention strategies. Included are 1) definition of risk factors or biomarkers that may be used in selecting cohorts and as endpoints for chemoprevention studies, 2) criteria for selecting risk biomarkers as endpoints and high-risk cohorts for chemoprevention studies, and 3) studies at major target sites that incorporate cancer risk-based measurements in their design.

CANCER RISK BIOMARKERS

For chemoprevention, the important cancer risk factors are those that can be measured quantitatively in the subject at risk. These factors can be called risk biomarkers and can be used to identify cohorts for chemoprevention. Those modulated by chemopreventive agents may also be used as endpoints in chemoprevention studies. Generally, the risk biomarkers fit into categories based on those previously defined by Hulka [9]: 1) carcinogen exposure, 2) carcinogen exposure/effect, 3) genetic predisposition, 4) intermediate biomarkers of cancer, and 5) previous cancers (Table I).

Carcinogen exposure biomarkers, measuring the presence of carcinogen in tissue or body fluid, include chemical mutagens and carcinogens, and viruses such as human papilloma virus (HPV) associated with cervical cancer [10] and hepatitis B virus (HBV) associated with liver cancer [e.g., 1]. For example, in these proceedings, De Flora describes molecular dosimetry methods for detecting urinary mutagen levels as an endpoint in chemopreventive intervention studies of *N*-acetyl-*l*-cysteine (NAC) in smokers [see also 11]. One result of these studies was that 600–800 mg NAC/day significantly reduced the mutagenicity of smokers' urine extracts in the Ames *Salmonella* assay.

Besides detecting the presence of carcinogen, carcinogen exposure/effect biomarkers provide evidence that carcinogen is interacting with tissue, typically at the molecular level, in a way that produces cancer. Such biomarkers are usually the result of very early carcinogen-DNA reactions. For example, Kensler and his colleagues have evaluated the correlation of rat liver tumor induction to urinary DNA adducts with the carcinogen aflatoxin B_1 (AFB_1). As will be described below and elsewhere in these pro-

TABLE I. Risk Biomarkers in Cancer Chemoprevention*

Risk biomarkers in chemoprevention are measures of cancer potential. This risk is normally quantified as relative risk (RR) once validated studies are done. Included are
Carcinogen exposure
e.g., Urinary mutagens, HPV or HBV infection, plasma hormone levels
Carcinogen exposure/effect
e.g., Carcinogen-DNA adducts, hydroxyguanosine residues
Genetic predisposition
e.g., APC, BRCA1, BRCA2, MLH1, MSH2, Li-Fraumeni syndrome (p53 mutation), ataxia telangiectasia, xeroderma pigmentosum, genetic polymorphism in carcinogen metabolizing enzymes (NAT1, NAT2, CYP450IAI, GSTM1, GSTP1, SRD5A2), mutagen sensitivity
Intermediate biomarkers
e.g., Intraepithelial neoplasia (histopathology including nuclear/nucleolar morphometry and ploidy in CIN, PIN, DCIS, colorectal adenomas, dysplastic oral leukoplakia, bronchial dysplasia, superficial bladder cancers, actinic keratosis), hyperproliferation, proliferation kinetics, genomic instability, oncogene overexpression/tumor suppressor loss, growth factor and growth factor receptor overexpression (e.g., EGFR), differentiation biomarkers (e.g., G-actin, cytokeratins, blood group antigens), biochemical changes (PSA levels)
Previous cancers/percancerous lesions
e.g., breast, bladder, head and neck cancers, colorectal cancers and adenomas
Risk biomarkers can be used to identify clinical cohorts for chemopreventive intervention. In some cases, risk biomarkers that are modulatable by chemopreventive agents may be used as endpoints in clinical chemoprevention studies

*Abbreviations: CIN = cervical intraepithelial neoplasia, DCIS = ductal carcinoma in situ, EGFR = epidermal growth factor receptor, HBV = hepatitis B virus, HPV = human papilloma virus, LCIS = lobular carcinoma in situ, PIN = prostatic intraepithelial neoplasia, PSA = prostate specific antigen.

ceedings, AFB_1-DNA adducts are inhibited by the chemopreventive agent oltipraz, and a current Phase II clinical chemoprevention trial with oltipraz is using these adducts as an endpoint. Also in these proceedings, Glickman reviews the use of mutational specificity—i.e., the evaluation of mutational spectra and "hotspots"—in evaluating the mechanisms of carcinogenesis from environmental exposures. Studies in his laboratory have focussed on mutations in the HPRT gene of peripheral T-lymphocytes. He also cited the database accumulated on human p53 gene mutations and the association of mutational hotspots in these genes with specific carcinogens (e.g., G→T transversion at codon 249 induced in liver by AFB_1 [12,13] and in lung cancers by radon [14]).

Genetic predisposition includes well-characterized germline mutations, many of which are associated with loss of tumor suppressor functions. Examples are APC (familial adenomatous polyposis leading to colorectal cancer) [15,16], BRCA1 and BRCA2 (breast and ovarian cancers) [e.g., 17–22], and p53 mutation resulting in Li-Fraumeni syndrome (multiple cancers including breast, colorectal, brain and leukemia) [3]. Several cancer-predisposing genes are thought to affect the ability of cells to repair carcinogen-induced damage. Prominent among these are the MLH1 gene on chromosome 3p and the MSH2 gene on chromosome 2p, which have been linked to hereditary nonpolyposis colon cancer (HNPCC) [23,24]. Also, recent cancer epidemiology and pharmacogenetic studies have suggested the importance of genetic polymorphisms affecting the ability to detoxify carcinogens [reviewed in 25,26]—e.g., glutathione *S*-transferase (GSTM1, GSTM2, GSTP1), *N*-acetyltransferase (NAT1, NAT2), cytochrome P450 (CYP450IAI), and steroid 5α-reductase type II (SRD5A2). In these proceedings, Henderson reviews these polymorphisms and outlines his studies with SRD5A2, androgen receptor polymorphisms, and their implications in susceptibility to prostate cancer. He also discusses recent work on genetic polymorphisms affecting susceptibility to breast cancer. Usually genetic lesions by themselves do not provide appropriate endpoints for chemoprevention studies, since they are not easily modified by chemopreventive agents and are distal in time and progression from the cancer. However, as will be described below, their presence identifies cohorts for chemopreventive intervention, and in association with other biomarker abnormalities can be useful in defining cohorts for Phase II and III clinical chemoprevention trials.

Mutations and changes in expression of tumor suppressors during carcinogenesis are also important. Particularly, Harris and colleagues have reviewed the associations of p53 changes with cancer [27,28]. Likewise, oncogenes and growth factors, which are activated by mutation or are overexpressed during carcinogenesis (e.g., *ras*, EGFR, c-*erb*B2), are significant genetic lesions in cancer [reviewed in 29]. Further, as described by Weinstein in these proceedings, mutations in cyclin and cyclin-related genes implicated in control of cell cycle progression may be predictors of cancer risk. Although it is not likely that any of these lesions will be eradicated by chemopreventive agents, their presence and activity may be decreased by damping the signal transduction pathways in which they participate, thereby selecting against proliferation of cells containing the lesions. Moreover, subjects such as smokers who are at risk for induction of these effects may be good candidates for chemopreventive intervention with antimutagens.

Besides these specific genetic lesions, general indicators of genetic susceptibility have been developed. For example, in these proceedings [see also 30] Spitz describes mutagen sensitivity as measured by bleomycin-induced DNA break frequency in lymphocytes in vitro. In lung cancer patients, 50% of cases tested had mutagen sensitivity scores 1 break/cell compared with 22% of controls.

Intermediate biomarkers of cancer particularly useful as risk biomarkers are intraepithelial neoplasia (IEN). These lesions are essentially precancers, are directly on the causal pathway to cancer, and their presence puts carriers at high risk for invasive disease [7,8, 31–33]. Several articles in these proceedings characterize IEN and describe their use in chemoprevention. For instance, Lagios defines a classification of ductal carcinoma in situ (DCIS) for selecting cohorts for chemoprevention studies in breast cancer. Baron and Burt review colorectal adenomas as a risk factor for selecting cohorts and as an endpoint for chemoprevention trials, and Bostwick similarly considers prostatic intraepithelial neoplasia (PIN). Re-

gression and prevention of recurring IEN are logical endpoints for chemoprevention trials. As will be described below in greater detail, current or previous IEN have already been used to define cohorts for clinical chemoprevention studies.

Other intermediate biomarkers may occur within IEN. Besides the genetic biomarkers described above, other changes associated with carcinogenesis are also being evaluated. For example, in these proceedings Hemstreet describes G-actin as a differentiation biomarker associated with bladder carcinogenesis [see also 34,35]. Tockman considers the appearance of p31 antigen, as well as genetic damage such as microsatellite instability, in sputum samples from patients with premalignant lung lesions [see also 36,37]. Bagg suggests approaches to identifying biomarkers of early hematological malignancies. Crawford and DeAntoni define the increasing levels of prostate specific antigen (PSA) in prostate carcinogenesis, and Coffey has discussed nuclear matrix proteins (e.g., PC-1, which appears, and NBP-2 and NBP-4, which disappear) [38] and increasing telomerase activity during prostate carcinogenesis [39].

Previous cancers in many targets such as head and neck, breast and bladder put patients at high risk for recurrence and new primaries. In a trial in head and neck cancer patients, for instance, the incidence of second primaries was 31% during a four-year follow-up period [40,41]. In breast cancer patients, the incidence rate of contralateral breast cancer has been estimated as 0.8%/year [42]. Superficial bladder cancers recur in 60–75% of patients within 2–5 years of treatment [43,44]. Like subjects with precancerous lesions, these patients can be good cohorts for chemoprevention studies. For example, in the head and neck cancer trial just cited, which is Hong's vanguard chemoprevention study, treatment with 13-*cis*-retinoic acid reduced the incidence of second primaries to 14%.

CRITICAL ASPECTS IN DESIGNING CHEMOPREVENTION STUDIES WITH RISK BIOMARKERS

Concepts fundamental to evaluating the use of risk biomarkers in chemoprevention studies are (1) the importance of accumulated risk from one or many factors, (2) the stochastic, multipath mode by which these factors are acquired during carcinogenesis, and (3) the long time period required for carcinogenesis. For example, the Gail model [45] defines the contributions to accumulated breast cancer risk from family history, previous breast biopsies, age, parity, and age of menarche. Family history, presumably associated at least partially with genetic predisposition, is the predominant factor. Previous breast biopsies diagnosed as benign suggest the presence and persistence of possible precancerous lesions. One interpretation is that the other factors reflect accumulated estrogen exposure—the relative risk (RR) increasing directly with dose. Similarly, accumulated risk has been demonstrated in epidemiological studies on chronic smokers. In these studies, the RRs for lung cancer repeatedly show a dose-response to the number of pack/years of smoking [reviewed in 46].

Fearon and Vogelstein have described the multipath process of carcinogenesis in colorectal cancer [15]. They have identified the lesions that contribute to cancer risk; namely, germline mutations such as APC, *ras* mutation and overexpression, hypermethylation, and loss of heterozygosity in chromosomes 17p (i.e., loss of p53 function) and 18q. The relevance of their model to this discussion of accumulated risk is that multiple lesions are required for cancer development, not all the lesions are seen in every cancer, and the same lesions are not seen in all cancers.

Carcinogenesis can require 20–40 years [e.g., 47–52]. Thus, even in very high risk cohorts cancer incidence may be a difficult endpoint to evaluate, requiring very large study populations and long study durations. For example, in the epidemiological studies in smokers cited above, the RRs for lung cancer are as high as 25; however, chronic exposure (for risk accumulation) is critical, and the incidence is still relatively low. It has been estimated that less than 20% of smokers will develop lung cancer in their lifetime [53]. The designs of two lung cancer chemoprevention trials show the impact of these factors on cohort size and study duration. A trial of β-carotene and vitamin E in Finnish male smokers was carried out in 29,000 subjects treated for 5–8 years [54]. The CARET trial of vitamin A and β-carotene planned to accrue 13,000 chronic smokers for a mean six years of treatment [55]. The importance of accumulated risk is shown by another group in this study. With a second risk factor of asbestos exposure added, the number of subjects required dropped to 4,000. However, this cohort is

still very large and not feasible for many chemoprevention efficacy studies.

The implications of these concepts for the risk biomarkers in chemoprevention clinical trials are summarized in Table II [see also 33]. The first criterion is that risk biomarkers fit the expected biological mechanism(s) of carcinogenesis in the target tissue. That is, the closer the association of the risk biomarker(s) to the cancer or the higher the accumulated risk associated with the biomarker(s), the higher the likelihood that the biomarker(s) will be useful as endpoints and in selection of cohorts for chemoprevention studies. A corollary is that panels of biomarkers representing the various possible carcinogenicity pathways may be better as endpoints and in defining high-risk cohorts. Also, it is desirable that chemoprevention trials be relatively short (e.g., Phase II trials are 1 month–3 years in duration; Phase III trials may be up to 10 years, but in many cancer targets $\leq$3 years duration should be feasible). Thus, for risk biomarkers used as endpoints, short latency compared with cancer is important. Further, for risk biomarkers defining cohorts, short latency between the appearance of the biomarker and subsequent cancer is needed.

The second and very important criterion is that the biomarker(s) and assay(s) provide acceptable sensitivity, specificity, and accuracy for evaluating chemopreventive efficacy. These factors ensure that a small trial will produce meaningful results. Several papers in these proceedings address the important issues in identifying risk biomarkers, particularly those indicating genetic susceptibility, and describe elegant techniques for assaying them. For example, Sidransky describes his laboratory's pivotal studies in the detection of clonal genetic alterations that identify tissues at risk for bladder, lung and head and neck cancers [see also 56]. Ronai discusses a sensitive PCR methodology that shows higher levels of K-*ras* mutations in sputum from lung cancer patients than in subjects without the cancer. Mao and Sidransky describe detection of microsatellite instability and loss of heterozygosity in urinary DNA from bladder cancer patients before these patients demonstrated positive urine cytology. As noted above, Tockman describes similar early biomarkers in sputum from patients with early dysplasia, and Anderson looked at overexpression and mutation of p53 in conjunction with expression of cytokeratins 8 and 18 in sputa as biomarkers of hyperplastic and early dysplastic lung lesions.

Gould characterizes an inherited pattern of allelic imbalance in rats that correlates to susceptibility for mammary gland cancer. This pattern may be a prototype molecular marker of cancer risk. You and colleagues describe the use of two-dimensional gel electrophoresis patterns to compare the genomes of cervical intraepithelial neoplasia (CIN) and cervical carcinomas with normal cervical tissue, and the use of confocal laser scanning microscopy (CLSM) to compare DNA content in normal cervical tissue and CIN III. In the gel electrophoresis study, different patterns were seen in normal and neoplastic tissue, and more changes from normal were seen in the cancers than in the precancerous CIN lesions. Similarly, the DNA index observed by CLSM was significantly higher in CIN than in normal tissue.

Also, Hittelman delineates the use of chromosome in situ hybridization to detect genetic changes in normal and precancerous tissue adjacent to cancers in lung, head and neck, bladder, cervix and breast. In these studies, the degree of genetic change detected correlated to histologic progression of the lesion toward cancer. Very importantly, approximately half the patients with premalignant lesions of the oral

TABLE II. Criteria for Selecting Risk Biomarkers in Identifying Cohorts and as Endpoints in Cancer Chemoprevention Trials

Fits expected biological mechanism
Differentially expressed in normal and high-risk tissue
On or closely linked to causal pathway for cancer
Latency is short compared with cancer
Biomarker and assay provide acceptable sensitivity, specificity, and accuracy
Assay for biomarker is standardized and validated
Statistically significant difference between levels in high- and low-risk groups
Relative risk (RR) has been quantified
Biomarker is easily measured
Biomarker can be obtained by non-invasive or relatively non-invasive techniques
Assay for biomarker is not technically difficult
For risk biomarkers used as endpoints
Modulated by chemopreventive agents
Biomarker modulation correlates with decreased cancer incidence
Dose-response effect of the chemopreventive agent is observed

cavity and high levels of genetic changes subsequently developed aerodigestive tract cancer.

A very interesting application is defined by Lipkin and his associates. They describe the *Apc*1638 mouse, which carries a mutation resembling that in human FAP and forms many gastrointestinal dysplastic lesions. Similarly, Jakoby and his associates [57] described the chemopreventive activity of the nonsteroidal antiinflammatory drug piroxicam on colorectal adenomas in another *Apc* mouse strain (Apc^{Min}). As these investigators have suggested, transgenic strains should facilitate the analysis of individual etiological and chemopreventive effects that may presumably be translated to clinical studies. In the National Cancer Institute chemoprevention program, we have been evaluating several other transgenic strains besides the *Apc* mice. These models are listed in Table III.

The third criterion of easy measurement applies to all biomarkers in chemoprevention trials, including those involving risk biomarkers. Biomarkers that can be sampled by non-invasive or relatively non-invasive methods, such as in blood or urine, have higher priority for development than those obtained by biopsy or other surgical procedures. This is particularly true for trials in healthier subjects where repeated biopsies would be beyond the limits of accepted medical care. On the other hand, in high-risk cohorts such as patients with previous colorectal adenomas or colorectal cancer, periodic biopsies are an established part of follow-up care. In such cases, biomarkers obtained in biopsy samples are of high priority, because of the opportunity to evaluate the histopathology in the high-risk tissue. Particularly important are measurements of changes in nuclear and nucleolar morphometry that reflect the progression from preneoplasia through IEN to cancer. We and others have previously described the ratio-

TABLE III. Transgenic Models in the NCI Chemoprevention Branch Testing Program*

Transgenic mouse model	Target	Agents	Genetic lesions	Histological lesions
Min	Colon	DFMO, piroxicam	Heterozygous Apc2549	Intestinal and colonic adenomas, some areas of CIS
Apc	Colon		Heterozygous Apc1638	Adenomas, adenocarcinomas
pim	Lymphatic system	DFMO, 4-HPR, oltipraz	Amplified pim-1	T-cell Lymphomas
TG.AC	Skin	DFMO, NAC	Ha-ras	Papillomas, possible carcinomas
TSG-p53	Skin	DFMO, NAC	Heterozygous p53 deficient	Papillomas, possible carcinomas
A/JxTSG-p53	Lung	PEITC	Heterozygous p53 deficient	Adenomas
A/JxUL53	Lung	PEITC	Heterozygous p53 mutant	Adenomas
v-Ha-ras	Skin	DFMO, 4-HPR, d-limonene, perillyl alcohol, piroxicam, all-trans-retinoic acid	Ha-ras + human keratin K-1	Hyperplasia, hyperkeratoses, squamous papillomas
C3(1)-SV40	Prostate	DHEA, 5α-reductase inhibitor, antiandrogen	Heterozygous rat prostatic steroid binding gene [C3(1)] + SV40 T-Antigen	Dysplasia, adenoma, adenocarcinoma
C3(1)-SV40	Mammary glands	DFMO, DHEA, vorozole	Heterozygous rat prostatic steroid binding gene [C3(1)] + SV40 T-antigen	Adenocarcinoma

*Abbreviations: DFMO = 2-difluoromethylornithine, DHEA = dehydroepiandrosterone, 4-HPR = all-trans-N-(4-hydroxyphenyl)-retinamide, NAC = N-acetyl-l-cysteine, PEITC = phenethyl isothiocyanate.

nale for these measurements and their quantification [e.g., 32,33,58–60]. Also, in Hittelman's study cited above, half the aerodigestive tract cancers that developed in patients with high levels of genetic changes were at sites distant from the oral cavity biopsy. This was seen by Hittelman as confirmation of the "field effect" in carcinogenesis [61,62]: high-risk tissue was detected in a wide area near, but not directly at, the cancer site. Very importantly, this result also suggests that simple biopsies in easily accessed tissues such as oral cavity may provide information for detecting cancer risk in nearby but less accessible tissues such as the other parts of the upper aerodigestive tract.

The special requirement for risk biomarkers used as endpoints in chemoprevention trials is obvious—expression of the biomarker must be affected by the chemopreventive agent being tested. Further, the biomarker modulation should correlate to decreased cancer incidence and show dose-response to the chemopreventive agent. As suggested above, this requirement has important implications for the feasibility of genetic lesions as endpoints. It is not likely that chemopreventive agents will eradicate a genetic lesion per se. However, a genetic lesion can be an endpoint in a chemoprevention trial if (a) cell populations containing the lesion are diminished by the chemopreventive agent in favor of normal cells, or (b) encoded proteins are modulated by the chemopreventive agent.

COHORTS FOR CHEMOPREVENTION TRIALS DEFINED BY RISK BIOMARKERS

The first column in Table IV lists representative cohorts at high risk for major cancers that are likely to benefit from chemopreventive intervention. The second column lists high-risk cohorts in these same major target sites that would be suitable for Phase II and III chemoprevention trials. As follows from the discussion above, the primary distinction between the two lists is the degree of accumulated risk. The higher the degree of accumulated risk and the closer the association of the risk to cancer, the higher is the feasibility and likely success of a chemoprevention trial in the cohort. The high rates of recurrence and new primaries in patients with previous cancers in targets such as head and neck, bladder, colon and breast suggest that such patients comprise suitable cohorts for chemoprevention trials [reviewed in 7]. The endpoints in these trials would be new lesions or earlier biomarkers of elevated risk. Similarly, patients with previous and current precancerous lesions, particularly IEN, make suitable cohorts for chemopreventive intervention studies. In the NCI Chemoprevention Branch clinical testing program, we are currently evaluating chemopreventive agents in Phase II trials in several groups of patients with precancerous lesions—patients with prostatic intraepithelial neoplasia (PIN), breast ductal carcinoma in situ (DCIS), colorectal adenomas, bronchial dysplasia, superficial bladder lesions (stage Ta, T1), CIN III, esophageal dysplasia, and dysplastic oral leukoplakia. Table V lists these trials. Many of the cohorts for future chemopreventive intervention listed in Table IV are genetic syndromes. Usually, these cohorts are not good candidates for chemoprevention trials, unless they have histological precancerous lesions, like many patients bearing APC mutations or hereditary nonpolyposis colorectal carcinoma genes (MSH1, MLH2, PMS1, PMS2) who develop early colorectal adenomas. The protocol design and statistical problems of using and evaluating high-risk cohorts where a predictive genetic test exists but precancerous lesions do not has been described recently by Schatzkin, Freedman and co-workers [63,64]. Although they acknowledged that there would be difficulties such as noncompliance of subjects who tested negative for the gene defect and the need for large cohorts, the investigators estimated that there could be savings in size, duration and cost for cancer prevention trials using genetic tests. These investigators also touched on the ethical issues that should be explored in developing clinical study protocols with such cohorts. Nayfield expands upon these issues in these proceedings, using BRCA1 carriers as an example. For instance, two fundamental concerns are the timing of genetic testing and disclosure of the results to the patient. There is concern that positive test results can prematurely limit ability to get health insurance or cause job loss, despite the lack of the more definitive risk of a pathologically evaluated lesion. Similarly, there is concern with falsely worrying the patient with the threat of cancer, where the risks are suspected but not confirmed and no standard of treatment exists. A conservative approach is that such lesions alone are not sufficient to warrant entry into chemoprevention studies. However, the possibility of additional risk biomarkers in these patients should be evaluated and could lead to

TABLE IV. Representative High-Risk Cohorts for Cancer Chemopreventive Intervention and Clinical Trials*

Target Populations for Intervention:	Cohorts for Clinical Trials:
Prostate	
PIN	PIN
Family history	Cancer on biopsy, treated by "watchful waiting"
PSA >3 ng/ml	High PSA
Prostatitis	Organ-confined prostate cancer, scheduled for prostatectomy (assess PIN and other biomarkers in whole gland)
Genetic polymorphism in testosterone activation (SRD5A2)	
Breast	
Genetic syndrome (e.g., Li-Fraumeni, BRCA1)	DCIS (intervention in presurgical period)
Family history	High-risk (family history, precancerous lesion, previous breast cancer) with multiple biomarker abnormalities
Previous breast, endometrial or ovarian cancer	
Precancerous lesions (e.g., atypical hyperplasia, DCIS, LCIS)	
Lung	
Tobacco use (smoking and chewing)	Chronic smoking with previous respiratory tract cancer and current bronchial dysplasia
Previous respiratory tract cancer	Chronic smoking or prior respiratory tract cancer
Bronchial dysplasia	
Genetic polymorphisms in carcinogen-metabolizing enzymes (e.g., CYP450IAI, GSTM2)	
Occupational exposures (e.g., asbestos, nickel, copper)	
Colon	
Genetic syndrome (e.g., APC, HNPCC)	APC or HNPCC and previous adenomas
Previous colorectal cancer or adenomas	Previous colorectal cancer or adenomas
Family history (colorectal cancer or adenomas)	
Previous breast or endometrial cancer	
Inflammatory bowel disease	
Bladder	
Previous superficial bladder cancers (Ta, T1)	Previous superficial bladder cancers (with or without TIS)
Tobacco smoking	
Occupational exposures (e.g., aromatic amines)	
Genetic polymorphism in carcinogen-metabolizing enzymes (e.g., NAT1, NAT2)	
Cervix	
HPV infection	CIN II or III
CIN	
Tobacco smoking	
Oral cavity	
Oral leukoplakia	Dysplastic oral leukoplakia
Tobacco use (smoking or chewing)	
Tobacco use with alcohol use	

*Abbreviations: CIN = cervical intraepithelial neoplasia; DCIS = ductal carcinoma in situ; HNPCC = hereditary nonpolyposis colorectal cancer; HPV = human papilloma virus; LCIS = lobular carcinoma in situ; PIN = prostatic intraepithelial neoplasia; PSA = prostate specific antigen; TIS = transitional cell carcinoma in situ.

definition of appropriate cohorts for early chemoprevention trials. The design of a trial involving high risk with associated intermediate biomarker abnormalities is described below.

DESIGN OF CLINICAL CHEMOPREVENTION TRIALS BASED ON RISK BIOMARKERS

From the discussion above and Table V, risk biomarkers clearly are already factors in the design of most clinical chemoprevention trials—particularly, in identification of cohorts. However, much additional critical thinking will be needed to fully integrate risk factors into chemoprevention studies. The article by Mark in these proceedings represents part of this process by delineating the implications biomarker incidence and rate of progression have in determining appropriate cohort size and study duration,

as well as stratification and weighting for analysis of results. Two recent protocols have also addressed some major issues on risk factors in design of chemoprevention trials.

In the first, both the cohort and the endpoint are based on the risk associated with exposure to an environmental carcinogen. Importantly, extensive effort was put into developing the assay for the risk biomarker endpoint and correlating this endpoint to cancer risk. This study, of the effect of the chemopreventive agent oltipraz against AFB_1- induced liver carcinogenesis, was designed by Kensler and colleagues, and is described briefly here and in detail by Kensler elsewhere in these proceedings [see also 65].

Epidemiologic studies have shown a strong association between estimated aflatoxin intake and primary liver cancer [e.g., 66]. High levels of aflatoxins, produced by *Aspergillus* species, have been found in groundnuts and maize in Africa, southeast Asia, and southern China, where these foods are dietary staples. In the chemoprevention study, groups of 80 subjects at high risk for liver cancer from AFB_1 exposure (and also from HBV_1 exposure) in the Qidong region of China were treated with oltipraz at 125 mg qd or 500 mg 1x/week for two months. Treatment was scheduled over the summer months, when AFB_1 exposure is highest. The primary endpoint of the trial is urinary AFB_1-DNA adducts.

Kensler and his colleagues developed the preclinical data supporting the trial design, which is as follows: (1) Oltipraz is a potent inducer of Phase II metabolic enzymes such as glutathione (GSH) *S*-transferase (GST); its chemopreventive activity has been attributed to enhancement of these enzymes resulting in increased conjugation and excretion of carcinogens. Studies in carcinogen-induced animals found lower levels of effective carcinogens in oltipraz-treated animals, as measured, for example, by carcinogen-DNA adducts; particularly, lower levels of AFB_1-DNA adducts have been observed [67,68]. (2) Oltipraz suppresses liver adenomas and carcinomas in AFB_1-treated rats [69]. (3) Liver tumor inhibition correlates to reduction of liver neoplastic foci, GST enhancement, and reduction of liver AFB_1-DNA adducts [69]. (4) Reduction of liver AFB_1-DNA adducts, in turn, correlates to reduction of urinary AFB_1-DNA adducts [70–72]. (5) And, weekly and twice weekly oltipraz doses were approximately as effective as daily 500 mg doses in reducing rat liver GST-positive neoplastic foci and increasing GST [72].

In the second study, which is also described in detail elsewhere in the proceedings, Fabian and coworkers have defined a risk-based cohort that is feasible for a short-term chemoprevention study [see also 73]. In this study, 213 women at high risk for breast cancer were selected based on having first-degree relatives with breast cancer (73%), prior biopsy indicating premalignant disease (26%), history of breast cancer (13%) or a combination of these factors (11%). Fine needle aspirates (FNA) from these women and 30 low-risk women were analyzed for cytological abnormalities and other biomarkers (aneuploidy, epidermal growth factor receptor (EGFR), estrogen receptor (ER), p53 and *erb*B-2) and compared. The results suggested that the presence of multiple biomarker abnormalities exclusive of cytology could be used to refine the selection of high-risk subjects. Thirty-one (31%) of the high-risk subjects had two or more biomarker abnormalities, while none of the low-risk group had more than one such abnormality. The presence of multiple biomarker abnormalities increased directly with cytologic atypia, ranging from 16% of subjects with normal cytology to 29% of those with hyperplasia to 60% of those with atypical hyperplasia. No significant differences in the number of biomarker abnormalities or abnormal cytology were seen among the original risk groupings (i.e., first-degree relatives, prior positive biopsy, history of breast cancer, or multiple factors). Because of the association of multiple biomarkers to cytological evidence of dysplasia, the investigators have suggested that changes in the pattern of biomarker abnormalities in the FNA (particularly, p53 and EGFR), as well as atypical hyperplasia, could be explored as endpoints in a chemoprevention study in this cohort.

PROSPECTS FOR RISK BIOMARKERS IN CHEMOPREVENTION

Throughout the discussion above, we have cited the challenges associated with using risk biomarkers as a basis for cohort selection and as endpoints in chemoprevention trials. The immediate hurdle is defining risk biomarkers that are highly predictive of cancer incidence, are quantitative, and can be used in short-term clinical trials. We are addressing this challenge conceptually by developing strategies based on accumulated risk—e.g., considering risks from

TABLE V. Current and Planned Phase II Clinical Chemoprevention Trials in High-Risk Cohorts*

Target site	Agent(s)	Cohort (treatment period)	Proposed endpoints
Prostate	DFMO DHEA 4-HPR	Scheduled for prostate cancer surgery (evaluation of biopsy tissue with associated PIN) (2–8 weeks)	Histopathology (PIN grade, nuclear/nucleolar polymorphism, ploidy), proliferation biomarkers (e.g., PCNA, Ki-67), differentiation biomarkers (e.g., $Lewis^Y$ antigen), genetic/regulatory biomarkers (e.g., TGFα, p53, bcl-2, pc-1, chromosome 8p loss)
	CATBN	High grade PIN, no carcinoma (3 years)	Histopathology (PIN grade and incidence, nuclear polymorphism, nucleolar size, ploidy), proliferation biomarkers (e.g., PCNA), genetic/regulatory biomarkers (e.g., TGFβ, altered oncogene expression), PSA
Breast	DFMO DHEA Exemestane 4-HPR Tamoxifen 4-HPR + tamoxifen	Mammographic lesion requiring biopsy (DCIS) (2–4 weeks)	Histopathology (DCIS grade, nuclear polymorphism, ploidy), proliferation biomarkers (e.g., PCNA, Ki-67, S-phase fraction)
	DFMO DHEA	High-risk with ≥2 biomarker abnormalities (p53, EGFR, Aneuploidy, ER, c-erbB-2) with or without atypical hyperplasia (6 months)	Histopathology (hyperplasia grade, ploidy), proliferation biomarkers (e.g., PCNA), genetic/regulatory biomarkers (e.g., p53, EGFR, ER, c-erbB-2)
Colon	Aspirin + calcium	Previous colorectal adenomas (6 months)	Proliferation biomarkers (PCNA), PGE_2 levels
	Sulindac Calcium Calcitriol Vitamin D_3	Previous adenomas (resected within past 2 years) or colon cancers (6 months)	Histopathology (nuclear polymorphism), proliferation biomarkers (DNA labeling index, crypt proliferation pattern—PCNA), differentiation biomarkers, genetic/regulatory biomarkers (p53, bcl-2)
	Sulindac sulfone	FAP patients (6 months)	Adenoma size and number, proliferation biomarkers (PCNA), genetic/regulatory biomarkers (apoptosis)
	Aspirin Folic acid Aspirin + folic acid	Previous colorectal adenomas (3 years)	Adenoma size and number
	Calcium + vitamin D_3	Colorectal adenomas <6 mm diameter (3 years)	Adenoma size and number, histopathology (nuclear/nucleolar polymorphism, ploidy), proliferation biomarkers (crypt proliferation pattern—PCNA)
	Sulindac	Colorectal adenomas (left-side, 5–9 mm diameter) (1 year)	Adenoma size and number, proliferation biomarkers (PCNA)
Lung	4-HPR	Chronic smokers with prior resected head/neck, lung, or bladder cancer who display bronchial squamous metaplasia (index ≥15%) or dysplasia (6 months)	Histopathology (dysplasia regression, ploidy), proliferation biomarkers (PCNA), genetic/regulatory biomarkers (p53, EGFR), mutagen sensitivity, micronucleated cell frequency

TABLE V. (Continued)

Target site	Agent(s)	Cohort (treatment period)	Proposed endpoints
	Oltipraz	Chronic smokers, or prior resected carcinoma of respiratory tract (6 months)	Histopathology (nuclear polymorphism, ploidy), proliferation biomarkers (Ki-67), genetic/regulatory biomarkers (p53), agent specific (GSTM phenotype, GST activity in lymphocytes, bronchial cells)
Cervix	DFMO 4-HPR	CIN III (6 months)	Histopathology (CIN grade, nuclear polymorphism, ploidy), proliferation biomarkers (PCNA), differentiation biomarkers (keratins, involucrin, transglutaminase), genetic/regulatory biomarkers (ras, EGFR, TGFα), agent specific (e.g., ODC activity, polyamine levels, RAR)
Bladder	DFMO	Previous superficial bladder cancer (Ta, T1 disease without TIS) (12 months)	Histopathology, proliferation biomarkers (Ki-67), differentiation biomarkers (Lewis^x antigen), genetic/regulatory biomarkers (EGF, EGFR, p53, PKC isotypes), agent specific (ODC activity, polyamine levels)
	4-HPR	Previous superficial bladder cancer (Ta, T1 disease with TIS, treated with BCG) (12 months)	Recurrence, histopathology (ploidy), proliferation biomarkers (Ki-67, DD23, M-344), differentiation biomarkers (G-actin)
Oral cavity	DFMO 4-HPR 13-cis-Retinoic acid	Dysplastic oral leukoplakia (6 months)	Recurrence, histopathology (dysplasia/leukoplakia grade, nuclear polymorphism, ploidy), native cellular fluorescence, proliferation biomarkers (PCNA, Ki-67, S-phase fraction), differentiation biomarkers (cytokeratin 19, blood group antigens), genetic/regulatory biomarkers (TGFβ)
Esophagus	DFMO	Dysplastic/metaplastic Barrett's esophagus (6 months)	Histopathology (nuclear/nucleolar polymorphism, ploidy), proliferation biomarkers (Ki-67), genetic/regulatory biomarkers (p53, TGFα, EGFR, microsatellite instability)
Skin	4-HPR	Actinic keratosis (6 months)	Histopathology (lesion grade), proliferation biomarkers (PCNA), genetic/regulatory biomarkers (EGFR, TGFβ)
Liver	Oltipraz	Aflatoxin exposure (Qidong, China) (2 months)	Urinary aflatoxin-DNA adducts, serum aflatoxin-albumin adducts

*Abbreviations: CATBN = chemopreventive agent to-be-named; CIN = cervical intraepithelial neoplasia; DCIS = ductal carcinoma in situ; DFMO = 2-difluoromethylornithine; DHEA = dehydroepiandrosterone; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; ER = estrogen receptor; FAP = familial adenomatous polyposis; GST = glutathione-S-transferase; 4-HPR = all-trans-N-(4-hydroxyphenyl)retinamide; ODC = ornithine decarboxylase; PCNA = proliferating cell nuclear antigen; PG = prostaglandin; PIN = prostatic intraepithelial neoplasia; PKC = protein kinase C; RAR = retinoic acid receptor; TGF = transforming growth factor; TIS = transitional cell carcinoma in situ.

lesions such as IEN which are on the causal pathway and closely resemble cancer, and including multiple risk factors in defining cohorts and endpoints. Technically sophisticated methods for characterizing risk, such as those based on quantitative analysis of chromosome damage patterns (e.g., electrophoretic genomic scanning, allelic imbalance, mutagen sensitivity, and quantitative measures of nuclear and nucleolar morphometry and cytometry) are also being evaluated. Very importantly, we have begun the process of developing the preclinical and pilot clinical data to support the design of short-term clinical trial protocols based on high-risk cohorts and risk biomarker endpoints.

As we stated above, cancer risk from lifestyle, occupational, environmental, and inherited causes defines the need for and the cohorts who will benefit from chemoprevention. One of the remarkable achievements in recent years has been the identification of genetic lesions that predispose subjects to cancer—these include both germline and acquired mutations leading to such cancer-promoting events as loss of tumor suppressor function, inability of cells to repair induced damage, overexpression of cellular growth and transcription factors, and inability to detoxify carcinogens. Although subjects with these lesions are not likely to comprise cohorts for Phase II and III chemoprevention trials unless they are expressing other biomarkers associated with carcinogenesis, these lesions suggest types of agents that may be effective and the cohorts to which chemoprevention will ultimately be directed.

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