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Intermediate Biomarkers of Precancer and Their Application in Chemoprevention

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Abstract The Chemoprevention Branch of the National Cancer Institute has established a program for the development of safe and effective cancer chemopreventive agents. This program includes identification of new agents, testing for efficacy *in vitro* and in animals, studies in animals to model clinical use, and preclinical toxicity and metabolism evaluation. Ultimately, the most promising agents progress to clinical trials. The long period required for cancer onset presents a significant challenge to the design of clinical trials for chemoprevention. Phase III trials in which cancer reduction is the endpoint require large subject groups (tens of thousands) and follow-up duration of more than five years. Because of these requirements, the costs of such trials are high. The Chemoprevention Branch is addressing this challenge by expansion of the preclinical and Phase II clinical efficacy efforts to include intermediate biomarkers of carcinogenesis as study endpoints.

The Chemoprevention Branch's studies focus on the development of biomarkers with high reliability and predictive value for cancer. Both single markers and batteries of complementary and parallel markers are evaluated. Among the criteria for biomarkers for chemoprevention studies are the following: (1) differential expression in normal and high risk tissue, (2) appearance early in carcinogenesis (the earlier a reliable biomarker appears, the greater is the chance for successful intervention with a chemopreventive agent), (3) high sensitivity, specificity, and accuracy relative to cancer, (4) ease of measurement (use of non-invasive techniques and small tissue samples is preferable), (5) demonstration of modulation by chemopreventive agents, and (6) correlation of modulation with decreased cancer incidence.

Key words: chemoprevention, clinical trial, drug development, intermediate biomarker

INTRODUCTION

The Chemoprevention Program of the National Cancer Institute is organized as a drug development program with clinical trials as the endpoint. The program is outlined in Figure 1, and has been described in detail previously [1,6, 7,11].

Briefly, chemopreventive drug development begins with the systematic identification of candidate agents. The newly identified agents are evaluated in a series of *in vitro* and animal screens to characterize their chemopreventive efficacy. Promising agents may be evaluated further in various animal models to evaluate the design of regimens for potential clinical testing and use. Candidates judged to have potential as human chemopreventives are subjected, as appropriate, to preclinical toxicity and pharmacokinetic evaluation. The most successful agents then progress into clinical trials.

Target populations for chemoprevention trials include persons at high risk for cancers because of such factors as genetic predisposition (*e.g.*, familial adenomatous polyposis) or exposure to carcinogens (*e.g.*, smokers). Other target populations include persons with known precancerous lesions (*e.g.*, cervical dysplasia) and patients with previously treated cancers. These target populations are considered to be relatively healthy. For most other types of drugs, the target population carries the disease being studied. Side effects can be tolerated more readily in patients for whom treatment is expected to relieve a disease than they can be in patients who are healthy as are those in chemoprevention trials. Also, in many clinical trials, the duration of treatment is limited to that needed to cure the disease, and, hence, is relatively short. In chemoprevention trials the ultimate endpoint is reduction in incidence or delay in onset of cancer — a process which can take many years. Thus, chemopreventive treatment is expected to be long-term, and with cancer as the endpoint, such trials require long treatment and follow-up periods and very large subject populations.

Despite these obstacles, there is a growing body of evidence demonstrating chemopreventive efficacy in the clinic. The Chemoprevention Branch itself currently is sponsoring

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approximately 40 Phase I, II, and III clinical trials. Recently, in one of these trials, Hong *et al.* [10] demonstrated that 13-*cis*-retinoic acid prevents the appearance of second primary tumors in patients previously treated for squamous cell carcinomas of the oral cavity and upper respiratory tract. Also, 13-*cis*-retinoic acid, though toxic at the doses used, was effective in preventing new skin cancers in patients with xeroderma pigmentosum [13].

CONCEPT OF INTERMEDIATE BIOMARKERS IN CHEMOPREVENTION RESEARCH

The concept of intermediate biomarkers is based on the long-accepted model of cancer as a multistage process. Epithelial neoplasia appears to be a continuum of clonal evolution initiated by a genetic alteration in a cell of histologically normal-appearing tissue; clonal expansion of the initiated stem cell; development of morphological abnormalities characteristic of dysplasia (intraepithelial neoplasia); and finally invasion across the basement membrane, *e.g.*, cancer [2 and this volume]. Thus, intermediate biomarkers are defined as morphological or biochemical alterations in epithelial tissue associated with a phase of carcinogenesis that precedes the "final" endpoint, cancer.

The use of the term "biomarker" to refer to additional types of biological alterations in carcinogenesis and cancer has caused some confusion in the literature. As shown in Table I, these include risk factors, exposure markers, drug effect markers, and tumor markers.

Risk factors are biomarkers which are not appropriate as modulatable endpoints in chemoprevention trials, but which confer higher risk for the development of carcinogenesis in certain individuals. These may be loosely categorized as lifestyle factors (*e.g.*, tobacco use), previously treated primary tumor, disease state (*e.g.*, ulcerative colitis), or genetic predisposition (*e.g.*, Li-Fraumeni syndrome) [16,17]. Of course, groups of individuals with these risk factors may also be monitored for subsequent appearance of modulatable intermediate biomarkers.

Exposure markers are essentially a type of risk factor. They include measures of carcinogen exposure such as the appearance of carcinogen-DNA adducts in blood. Like other risk factors, exposure markers are probably not appropriate endpoints for clinical chemoprevention studies. Also, like other risk factors, persons with these markers may be monitored for the appearance of modulatable intermediate biomarkers.

Drug effect markers indicate that a chemopreventive drug is producing an effect which may or may not be directly related to carcinogenesis. One example of such a marker is prostaglandin synthesis inhibition by nonsteroidal antiinflammatory drugs (*e.g.*, sulindac). Drug effect markers can be used in combination with intermediate biomarkers to demonstrate that the chemopreventive agent is pharmacologically active in the study group, whether or not it is efficacious as a chemopreventive.

Tumor markers are the conventional markers of the presence of cancer, such as carcinoembryonic antigen (CEA) [22]. Most of these occur too late in the carcinogenic process to be useful in chemoprevention.

THE NEED FOR INTERMEDIATE BIOMARKERS IN CHEMOPREVENTION RESEARCH

It is not unduly optimistic to assume that clinical research will begin to yield practical applications of chemopreventive agents in the reduction of cancer incidence by the beginning of the next decade. However, as suggested above, the time involved in the continued use of cancer incidence as the study endpoint is of concern. In the general population, cancer is a rare outcome; the overall yearly cancer rate is 3-4 cases per 1000 persons [cited in 23]. In studies of specific sites, the rate is even lower by 1-2 orders of magnitude. Consequently, the usual Phase III

TABLE I. Types of Biomarkers in Cancer

Cancer Marker	Description
Intermediate Endpoints	Biological alterations in tissue between initiation and tumor development. Includes premalignant lesions, histological changes, cell proliferation markers, cell differentiation markers, and genetic alterations leading to cancers.
Risk Factors	Lifestyle factors, disease states, genetic predisposition, previous primary tumor.
Exposure Markers	A subset of risk factors. Includes measures of carcinogen exposure such as carcinogen-DNA adduct formation.
Drug Effect Markers	Effects produced by a drug which may or may not be directly related to carcinogenesis. An example is prostaglandin synthesis inhibition.
Tumor Markers	Traditional markers appearing after cancer has developed, *e.g.*, CEA, α-fetoprotein.

trials require large sample sizes (tens of thousands), need follow-up duration in excess of five years, and incur high costs [21]. These problems are being addressed by expansion of the preclinical and Phase II efficacy effort to include modulation and validation of intermediate biomarkers of carcinogenesis as study endpoints. The role of the biomarker effort in chemoprevention drug development is indicated in Figure 1. Over the next several years this role will become increasingly important.

Intermediate biomarkers are biological alterations in tissue which "mark" or signal a stage of carcinogenesis between initiation and the development of a malignant tumor. Experimental studies will focus on the development of biomarkers with high reliability and predictive value as measured by such factors as specificity, sensitivity, and overall accuracy in correlating with cancer endpoints. An important concept in this regard is the potential utility of a battery of biomarkers, rather than a single biomarker, to approximate a cancer endpoint. Often, the results of a set of several complementary or parallel markers will be a more reliable predictor of a cancer than will a single marker. The methods for measuring biomarkers should lend themselves to the rapid screening of large populations. Ideally these methods should not be invasive, overly complicated, or too expensive.

APPLICATION OF INTERMEDIATE BIOMARKERS IN CHEMOPREVENTION RESEARCH

Intermediate biomarkers may be applied to chemoprevention research in two general ways. First, intermediate biomarkers, either singly or in a battery, may be used as study endpoints in clinical chemoprevention trials instead of the "final" endpoint, cancer incidence. Intervention with a putative chemopreventive agent should result in modulation of expression of the marker. The first successful example of this was the modulation of oral leukoplakia by 13-*cis*-retinoic acid. In a randomized, placebo-controlled, double-blind chemoprevention trial, Hong *et al.* [9] demonstrated that 3 months of retinoid treatment produced significant clinical regression as measured by lesion size, which was maintained for at least one additional month. Dysplasia, the histological marker used, also demonstrated regression.

Second, the presence of an intermediate biomarker or group of biomarkers can serve to identify high risk populations selected for chemoprevention trials. The cohort of individuals with demonstrated oral leukoplakic lesions mentioned above served as a study population at increased risk of malignant transformation. Additional current examples of intermediate biomarkers defining chemoprevention trial populations are cervical dysplasia, colonic adenomatous polyps, actinic keratosis, and bronchial squamous metaplasia.

Individuals with risk factors may also be monitored for the rate of appearance of related markers. Additional high risk populations currently being monitored in NCI-sponsored trials include asbestosis patients, individuals previously cured of basal cell carcinoma of the skin, and individuals with a genetic susceptibility for familial polyposis coli.

THE BENEFITS OF THE APPLICATION OF INTERMEDIATE BIOMARKERS IN CHEMOPREVENTION

The benefits of the application of intermediate biomarkers in chemoprevention are multiple (Table II). First, using a study endpoint correlated with earlier stages of carcinogenesis reduces the time interval necessary for the trial. Dr. Hong and co-workers [15] have demonstrated that time to modulation could be achieved in about 3 months in the case of oral leukoplakia. Second, efficacy trials require smaller study population sizes since the

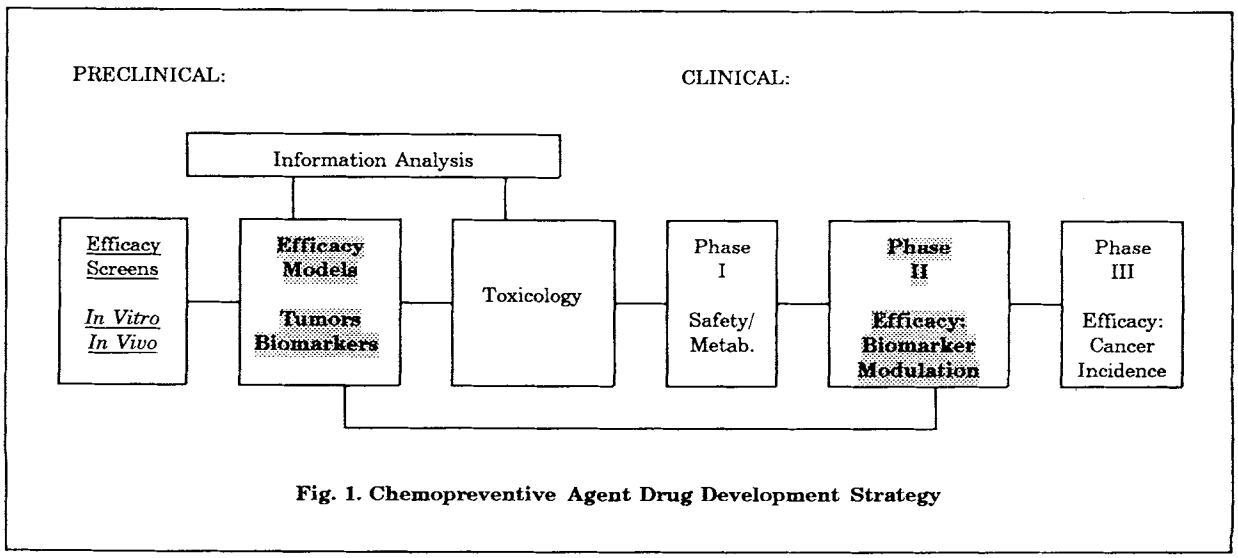

Fig. 1. Chemopreventive Agent Drug Development Strategy

Table II. Value of the Application of Intermediate Biomarkers in Chemoprevention

- Trials require shorter durations
- Trials require fewer subjects
- Trials are lower in cost
- Only small tissue samples required to monitor response
- Rationale provided for Phase III trials
- Effective dose determined for Phase III trials
- Basic scientific contributions to understanding mechanisms of carcinogenesis provided

outcome is less rare than cancer incidence. It has been suggested that a study of proliferation and diet in the large bowel might require several dozen subjects over a few months rather than thousands of subjects over a period of years [15]. Smaller sample populations may also be used when study populations are defined by risk markers. The third result, reduced cost with increased benefit, is a direct outcome of the first two considerations — less manpower and time are necessary to administer these studies. Fourth, modulation of intermediate biomarkers can be monitored definitively in smaller tissue samples. This is in part a result of the development of more specific detection and amplification methods such as DNA probes and the polymerase chain reaction [15]. Fifth, the results of Phase II efficacy trials assessing the modulation of intermediate biomarkers may serve as rationales for performing Phase III efficacy trials using cancer incidence as the endpoint. Phase III trials may, in turn, validate modulation of the marker as a predictor of reduced cancer risk. Sixth, Phase II trials using intermediate biomarkers could be used to determine the optimal dose of chemopreventive agents, the timing of administration, and possible confounding factors for Phase III efficacy trials [20]. Doses used in Phase I toxicity screening trials are not applicable to long-term efficacy trials in relatively healthy individuals [15]. Previous Phase III trial doses were based on informed guesswork; a more scientific basis such as that provided by trials using intermediate biomarkers is needed. Finally, those intermediate biomarkers which are the most closely associated with the process of carcinogenesis will contribute to our understanding of the mechanisms of cancer.

IDENTIFICATION, EVALUATION AND VALIDATION OF INTERMEDIATE BIOMARKERS AS ENDPOINTS

Before intermediate biomarkers can be used as endpoints in chemoprevention studies they must be validated. A number of basic criteria are considered when evaluating the potential of a marker to predict carcinogenic risk and to serve as an intermediate endpoint for clinical chemoprevention studies. Some of the questions to be considered in evaluating a marker are listed below. As noted above, it is expected that batteries of markers will sometimes provide more reliable and better-validated endpoints for chemoprevention studies than will single markers. The criteria below apply to batteries of markers as well as to single markers.

Is the intermediate biomarker differentially expressed in normal and high risk tissue? Can the progression from normal tissue to marker to cancer be established in a temporal fashion?

At what stage of carcinogenesis does the marker appear? The earlier a reliable marker appears in the carcinogenic process, the greater is the chance for successful intervention with resultant decreased cancer risk. Premalignant lesions, *i.e.*, neoplastic lesions that are intraepithelial and preinvasive, are well-established precursors of cancer development and, as mentioned previously, a number of studies have investigated intervention at this level. However, an ultimate goal should be to identify biochemical and genetic alterations which occur at earlier stages in the carcinogenic process.

Does the marker and its assay provide acceptable sensitivity, specificity and accuracy? How reproducible are the preclinical and clinical experiments demonstrating the relationship between the marker and carcinogenesis? Is the marker sensitive for carcinogenesis. That is, does it appear with high frequency in precancerous or high risk tissue? Is the marker specific for cancer? Does it increase in high risk tissue, but not in response to other conditions, such as disease or wound healing?

How easily can the marker be measured? Markers that are measurable by non-invasive techniques (*e.g.*, mucosal brushing, sputum, urine) and in small tissue specimens will be easier to obtain and are thus superior for use in chemoprevention studies. This may prove to be an extremely important consideration for monitoring healthy individuals.

Can the marker be modulated by chemopreventive agents? Does preclinical or early clinical data suggest that the intermediate biomarker is modulated by chemopreventive agents?

Does modulation of the intermediate biomarker correlate with a decrease in cancer rate? The validation of intermediate biomarkers as endpoints in chemoprevention research involves correlation of their modulation with a decrease in the rate of a related cancer. Only then can they be used as replacement endpoints in chemopreventive drug development. The statistical considerations in validating intermediate endpoints are discussed by Freedman in this volume.

INTERMEDIATE BIOMARKERS IN THE COLON AS A MODEL

This workshop focuses on the identification, evaluation and validation of potential intermediate biomarkers in the colon as a model system. This organ was selected for several reasons. First, colon cancer accounts for 14% of all cancers in the U.S. in both males (third highest incidence by organ) and females (second highest incidence by organ) [3]. Second, the histological sequence of development of the majority of human colon cancers is well-established; cancers are generally believed to have developed from adenomas in the so-called "adenoma-carcinoma sequence" [4]. Third, correlation of genetic and biochemical alterations with the appearance of adenomas has led to the identification of a number of additional intermediate biomarkers in the colon [14]. Since adenomas may be further graded as to malignant potential [19,18], the value of specific genetic and biochemical biomarkers in predicting the risk of malignant transformation may also be determined. Finally, the various stages of carcinogenesis are accessible and obtainable for study.

Potential intermediate biomarkers in the colon can be divided into general classes including histological and premalignant lesions, and proliferation, differentiation, and genetic biomarkers (Table III).

Histological Biomarkers

The normal colon is composed of test-tube shaped glands, the crypts of Lieberkuhn, which open at the mucosal surface. A balance between dividing cells in the

TABLE III. Examples of Intermediate Endpoint Colon Biomarkers by Class

Histological Biomarkers

- Adenomatous Polyps (Intraepithelial Neoplasia)
- Focal Epithelial Dysplasia (Ulcerative Colitis)
- Aberrant Crypts

Proliferation Biomarkers

- Expansion of the Proliferative Compartment
  - (*e.g.*, ^{3}H-Thymidine Labelling, BrdU Uptake, S-Phase Fraction, PCNA, Ki-67)
- Increased Ornithine Decarboxylase (ODC) Activity

Differentiation Biomarkers

- Abnormal Blood Group-Related Antigens (*e.g.*, Le^{x}, Le^{y})
- Abnormal Mucin Core Antigens (T, Tn, Sialyl Tn Antigens)
- Apomucins (MUC 1, 2, 3 Genes)
- Cytokeratins
- Brush Border Membrane Enzymes
  - (*e.g.*, Sucrase/Isomaltase)

Genetic Biomarkers

- Abnormal Gene Expression Patterns
  - (*e.g.*, Decreased Cytochrome Oxidase Subunit 3)
- Altered Cellular DNA Content
  - (*e.g.*, Aneuploidy, DNA Index)
- Chromosomal Structural Changes
- Altered DNA Methylation
- Activated Oncogenes (*e.g.*, c-*ras*, c-*myc*, c-*src*)
- Deleted Tumor Suppressor Genes and Other Chromosomal Losses (*e.g.*, FAP gene, APC gene, chromosomes 17, 18)

deepest third of the crypts and migration and exfoliation at the surface maintains the flat-appearing surface of the colon. A change in this balance may result in the formation of an adenomatous polyp, an intraepithelial neoplastic lesion exhibiting dysplasia. Adenocarcinomas of the colon are typically derived from adenomatous polyps. One major exception to the adenoma-carcinoma sequence is the occurrence of colorectal cancer in inflammatory bowel disease, especially ulcerative colitis. However, although the lesion is flat, the histological changes are generally the same [18]. These lesions will be discussed by Hamilton, Levin, Wargovich, and Pretlow in this volume.

Proliferation Biomarkers

One of the hallmarks of both malignant and premalignant lesions is abnormal cellular proliferation. Thus, indicators of proliferation may be important intermediate biomarkers for chemoprevention research. Examples include measurement of expansion of the proliferative compartment by thymidine labelling, BrdU uptake or PCNA histochemistry; enhanced ornithine decarboxylase activity; and expression of thymidine kinase. These intermediate markers will be described by Lipkin, Biasco, and Risio in this volume.

Differentiation Biomarkers

As cells differentiate, a specific pattern of expression of cellular components, such as proteins and carbohydrates, occurs. Since cancer cells undergo aberrant patterns of differentiation, it is likely that cellular components characteristic of differentiation will be modified during the progression of intraepithelial neoplasia. For example, during the development of increasingly severe dysplasia, the expression of certain cell surface or secreted carbohydrate conjugates may be altered. These include blood group antigens, mucins, and cytokeratins [8,12]. These intermediate markers are discussed by Kim, Itzkowitz, Boland, Ho, and Coffey in this volume.

Genetic Biomarkers

The accumulation of genetic changes within a single cell has been theorized to be responsible, at least in part, for the development of cancer. The importance of genetic instability is illustrated by the induction of mutations and chromosomal aberrations by most carcinogens, the detection of karyotypic variation in many solid tumors, and a higher incidence of cancer in individuals with decreased DNA repair syndromes. The more "gross" changes include alterations in cellular DNA content (aneuploidy, DNA index), nuclear aberrations, and altered patterns of gene expression. These aspects are addressed by Hittelman, Ahnen, and Augenlicht in these proceedings.

The more specific genetic alterations include activation of cellular oncogenes via point mutations, rearrangements or amplification; or germ line or somatic inactivation of tumor suppressor genes through point mutations or allelic or chromosomal deletions. Fearon and Vogelstein [5] have proposed a genetic model for colorectal tumorigenesis which incorporates the adenoma-carcinoma sequence. They theorize that an accumulation of genetic alterations is necessary for the formation of malignancy, although a preferred sequence does not appear to occur. Thus, detection of early stages of transformation at the genetic level may be possible using antibodies against secreted mutant gene products or detection at the DNA level. Articles in this volume by Cho and Cooper will discuss these intermediate biomarkers.

COMPLIANCE WITH THE FEDERAL REGULATORY PROCESS

The final step in developing chemopreventive drugs is successful compliance with the regulatory process. The FDA presently is considering a program for approval of new drugs at the earliest possible point at which safety and efficacy can be established. This program relies on the effect of a drug on "surrogate markers" of disease. The modulation of validated intermediate biomarkers of precancer could qualify as demonstration of the efficacy of drugs as cancer chemopreventives.

CONCLUSIONS

Cancer typically takes many years to develop. It may be possible to halt or at least prolong the carcinogenic process by intervening with chemopreventive agents at numerous points during the precancerous stages of intraepithelial neoplasia. Continuing advances in basic knowledge and analytical methods of molecular biology will facilitate the development of successful intervention techniques. Above all, the identification and validation of intermediate biomarkers as surrogate endpoints for chemoprevention trials will greatly accelerate the testing of these interventions in clinical trials and shorten the time and cost required to establish effective chemopreventive agents.

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