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What is Chemoprevention and How Can Surrogate Endpoint Biomarkers Shorten Clinical Trials

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The Chemoprevention Program of the National Cancer Institute, National Institutes of Health, USA, is developing a number of drugs which inhibit the progression of preinvasive neoplasia. The NCI is supporting 17 different clinical trials of chemopreventive agents in major organ systems which use the following surrogate endpoint biomarkers: ploidy change, proliferative rate change, change in nuclear and nucleolar morphometry (size, shape, texture), and change in nuclear pleomorphism. A "surrogate endpoint biomarker" is defined as a change in early preinvasive intraepithelial neoplasia (including dysplasia), produced by treatment with a chemopreventive agent, which closely predicts that the agent will block progression of preinvasive neoplasia to invasive cancer, i.e., that it will produce a decrease in cancer incidence. Four NCI-sponsored large-scale chemoprevention trials that are in progress. Two are using micronutrients: (a) β-carotene alone. (b) β-carotene and retinol, and two are testing the efficacy of tamoxifen in preventing breast cancer and finasteride in preventing prostate cancer. Now in Phase I clinical trials are four NSAIDS (piroxicam, ibuprofen, aspirin, and sulindac), DFMO, carbenoxolone, oltipraz (a dithiolthionc), and the combination of DFMO with piroxicam. Showing efficacy in animal models and being tested for toxicity are ellagic acid, phenhexyl isothiocyanate, curcumin, perillyl alcohol, S-allylcysteine, N-acetylcysteine, fluasterone (16 α-fluorodehydroepiandrosterone), and the combinations 4-HPR plus oltipraz and 4-HPR plus tamoxifen. In planning for the continuous identification and development of new chemopreventive compounds, it has been found useful in practice to classify chemopreventives as either antimutagenic or antimitogenic. Antioxidants, because of their similar mechanism of action, have been grouped separately as a third class. Antioxidants are both antimutagenic and antimitogenic.

Key Words: Chemoprevention, Endpoint biomarkers, Clinical trials

What is cancer chemoprevention?

Cancer chemoprevention is the prevention of clinical cancer by giving drugs or dietary constituents prior to or during the early phases of precancerous neoplasia, i.e., while the neoplastic process is still confined to the intraepithelial

compartment and has not yet become invasive. Chemoprevention includes the prevention and treatment of genomic instability, the earliest alteration in the carcinogenic process, which begins in normal appearing epithelium prior to the occurrence of dysplasia and long before invasiveness. Genomic instability is a condition of the cellular DNA characterized by ever-expanding structural abnormalities and mutations; it is produced by exposure to carcinogens from the environment or by in is produced by exposure to carcinogens from the environment or by inherited abnormalities of DNA repair, such as those found in Fanconi's Anemia, Ataxia Telangiectasia, or Xeroderma Pigmentosum. The major criterion for the diagnosis of cancer is invasiveness, or impending invasiveness evidenced by full-thickness severe dysplasia (also called carcinoma in situ). Thus, the treatment of intraepithelial neoplasia with drugs can be viewed either as the chemoprevention of neoplastic progression from the intraepithelial, precancerous state to the invasive, cancerous state, or as the chemotherapy of intraepithelial neoplasia.

How do cancer chemopreventive drugs work? Ans: by slowing or stopping neoplastic clonal evolution

1) What is clonal evolution?

Clonal evolution is the continuous appearance within a neoplastic cell population of mutant cells able to escape ambient growth control mechanisms and form clonal expansions which compete with each other on the basis of fastest growth rate[1,2]. Vogelstein[3] has revealed an outstanding demonstration of clonal evolution in adenomatous polyps of the colon: he showed that during the progression of intraepithelial neoplasia in the polyps, there occurs a series of genetic lesions, each of which is associated with a wave of overgrowing clonal cells.

Clonal evolution is the underlying mechanism by which neoplasms tend to develop ever-greater structural and functional diversity. The emergence of resistant clonal cell variants out of this diversity is the means by which neoplasms escape normal growth controls and also become resistant to chemotherapeutic agents and radiotherapy. Frequently, more than one genetically altered clone is found in different parts of a tumor, either by direct visualization or by genetic analysis. The different histological patterns used by the pathologist to construct the Gleason score in prostate cancer, for example, are derived from separate clonal expansions of cells.

How do chemopreventive drugs slow or stop clonal evolution?

This question requires a few background comments before it can be answered. Combustion products of cigarettes and fossil fuels contain both mutagenic and proliferation-inducing mitogenic molecules, as well as irritant molecules that induce epithelial proliferation associated with reactive inflammation. It is a striking fact that the number of years before lung cancer appears after starting to smoke is inversely related to the number of cigarettes smoked per day[4]. Interpreted at the cellular level, this observation indicates that during the progression of intraepithelial neoplasia induced in the respiratory mucosa by concurrent exposure to mutagenic and mitogenic molecules in cigarettes smoked per day, the faster is the rate of clonal evolution to the point of invasive neoplasia, i.e., cancer. This example in smokers illustrates the general principle, amply confirmed

by animal experiments[5], that the rate of clonal evolution in human carcinogenesis is continuously driven by the dose level of concurrent exposure to mutagens and mitogens found in the environment and endogenously[5].

Thus, chemopreventive drugs work by suppressing the two concurrent driving forces of neoplastic clonal evolution: mutagenesis and mitogenesis[7]. Accordingly the are categorized as being either antimutagens, antimitogens, or both.

Environmental oxidants, such as the organic oxides and peroxides found in cigarette smoke of fossil fuel exhausts, and prooxidants, which are molecules that cause increased production in cells of superoxide, hydrogen peroxide, and hydroxyl free radicals, are both mutagenic and mitogenic. The antioxidant chemopreventive agents that block them form a category of chemopreventives that are both antimutagenic and antimitogenic. Finally, the nonsteroidal antiinflammatory agents (NSAIDS) form a category of chemopreventives that work by blocking the arrival at an inflammatory site of monocytes, macrophages, and neutrophils producing extracellular superoxide and peroxide. NSAIDS are essentially antioxidant, and therefore are both antimutagenic and antimitogenic in their action. One example of an antimutagen is oltipraz, a dithiolethione, which induces glutathione-S-transferase in tissues, thereby accelerating the reaction of glutathione with activated carcinogens that results in their inactivation and excretion. An example of an antimitogen is tamoxafen, which blocks the stimulation of proliferation by estrogen in estrogen-sensitive tissues. Examples of antioxidants are vitamins C, E, and the many plant phenolics found in green vegetables such as the flavonoids, all of which react with oxidant molecules to form stable, non-reactive compounds that "soak up" or scavenge free radical electrons[6]. Finally, aspirin is an example of a category of non-steroidal antiinflammatory agents which, as a rule, are cancer chemopreventive in animal models, and, in the case of aspirin, likely in humans as well[8].

The sharp contrast between drugs used for chemoprevention and drugs used for cancer chemotherapy should be noted.

Chemopevention uses drugs that are antimutagenic, whereas chemotherapy uses the highly promutagenic alkylating agents such as nitrogen mustards and nitrosoureas. Chemoprevention uses antioxidants, whereas chemotherapy uses highly prooxidant antibiotics, such as doxorubicin, bleomycin, and mitomycin C, which are redox cyclers that generate mutagenic and cytotoxic free radical oxygen.

What are surrogate endpoint biomarkers (SEBs), and why are they needed in clinical trials of chemopreventive agents?

Surrogate endpoint biomarkers (SEBs) are molecular, cellular, or tissue changes associated with the neoplastic process which occur prior to the stage of invasiveness, i.e., during the stage of intraepithelial neoplasia (see Fig. 1), and which exhibit a measurable response to chemopreventive agents that accurately predicts their effect on cancer incidence.

At present, a serious barrier to development of the field of chemoprevention is the unacceptable cost (millions of dollars), long duration (5 ~20 years), and large scale of effort (thousands of subjects) required by clinical trials which use the endpoint of cancer incidence reduction. It is urgent that the endpoint of cancer incidence reduction be replaced by surrogate endpoint biomarkers, which occur much earlier during the preinvasive phase of neoplastic develop-

ment. With such surrogate endpoints the trials can be carried out in year or less, and, depending on the precision of the assay for surrogate endpoints, may require fewer subjects.

What is the role of computer-assisted quantitative image analysis (CQIA) in improving the precision of seb assays?

The advent of computer-assisted cytometric techniques offers the promise of quietly revolutionizing the practice of diagnostic histopathology. The sensitive and precise measurements of the morpho-and photometric parameters of dysplasia made by the computerized image cytometer, when compared to the present-day alternative of subjectively estimating such categories as "moderate to sever", "pleomorphism", or hyperchromasia, offer increased diagnostic precision that is greater by many orders of magnitude.

Basically, two modalities are used in image analysis. One, cytomorphometry, measures geometric relationships, such as nuclear dimensions, chromatin texture, and nucleolar size, shape, and position. The other, cytophotometry, measures cell and nuclear optical density at different wave lengths after staining with different dyes. Since there exist innumerable tissue surrogate biomarker assays (see below) which depend on the amount of chromogen development fixed to a cell or nucleus by chemical, antibody, or cDNA probes that are marked by a second chromogen-generating molecule (e.g., a fluorescent dye or horseradish peroxidase), the range of applications of image cytophotometry is very wide.

What possible choices are there for SEBs that can be used in chemoprevention trials?

In choosing a SEB for use in chemoprevention clinical trials, a variety of structural alterations characteristic of neoplasia at every level of organization is available for consideration. At the genomic level, many potentially useful SEB are possible, based on the different types of genomic instability which occur during neoplasia (reviewed in 4). Examples are DNA single and double strand breakage processes, point mutations, mismatch repair mutations, microsatellite instability, gene amplification and allelic loss, and the karyotypic aberrations of aneuploidy and aneusomy. Activated oncogenes and inactivated tumor suppressor genes have frequently been considered as potential SEBs in spite of their relatively poor sensitivity; they are present in about only about 50% or less of common carcinomas (p53 is an exception, occurring in up to 70% of cases of colorectal carcinoma and 90% of pancreatic carcinoma). At the cytoplasmic level, aberrantly synthesized differentiation molecules offer promise as SEBs, e. g. aberrant glycosylation of glycoproteins, including the Ta, T, and sialo-T antigens[4].

The well-known growth factors PDGF, EGF, TGF, FGF, IGF, and their receptors, are possessed by epithelial cells as well as inflammatory cells[9]. Attempts to use growth factors/receptors as SEBs faces the serious problem of variable contamination of tissue preparations by inflammatory cells possessing the same growth factors/receptors, and adequate control of this variability may be difficult. At the cellular level, the proliferative index and nuclear/nucleolar morphometric parameters measured by computer-aided quantitative image analysis

appear to be useful SEBs; they are discussed in more detail below.

What are the best choices for surrogate endpoint markers?

The chemoprevention branch, national cancer institute, is now sponsoring 18 clinical trials of chemopreventive agents in breast ductal carcinoma in situ, lung dysplasia, colon adenomatous polyps, cervical intraepithelial neoplasia, bladder superficial neoplasia, and skin actinic keratoses. In each of these trials, the basic SEB used are those based on the morphologic and functional criteria used by the pathologist to make the diagnosis of intraepithelial neoplasia, measured by computer-aided quantitative image analysis. These diagnostic criteria are: increased nuclear size, abnormal nuclear shape, increased nuclear stain uptake, pleomorphism (increased variability of nuclear size, shape, and stain uptake), increased mitoses, abnormal mitoses, and abnormal or absent maturation. In glandular epithelia such as breast and prostate, an additional criterion is the presence of an increased number of nucleoli showing enlargement, abnormal shape, and plemorphism of size and shape. SEBs based on these criteria, and measured by computeraided quantitative image analysis, are: proliferative index, ploidy status, nuclear morphometric parameters of increased nuclear size, altered nuclear shape, altered nuclear texture, abnormal nuclear variation in size, shape, and texture, and nucleolar morphometric parameters of nucleolar number, size, shape, position, and pleomorphism.

1) Proliferative index

The proliferative index is measured using antibodies against PCNA, Ki-67, Mid-1, and BrdU associated antigens, by tritiated-thymidine uptake/autoradiography, and by mitotic counts. The proliferative index has proven to be a reliable prognostic factor in breast cancer, accurately predicting the recurrence-free survival and overall survival either by itself[10], or as part of a commonly used grading system[11].

2) Ploidy status

Aneuploidy has been shown to occur during intraepithelial neoplasia in bladder[12], prostate[13,14], breast[15~18], and cervix, skin, oral leukoplakia, larynx, lung, esophagus, stomach, and colo-rectum (reviewed in 19). In one study of breast DCIS, the cribriform pattern exhibited 38% aneuploidy, whereas the comedo pattern exhibited 82% aneuploidy[17]. In another study, atypical hyperplasia of the breast also exhibited aneuploidy in 4 of 13 cases[18]. With regard to invasive neoplasia, ploidy status has proven to be a reliable prognostic factor in both breast[19,20], and prostate[21] cancer.

3) Nuclear morphometry (nuclear size, shape, texture, and pleomorphism)

Pleomorphism is measured by the pleomorphism index, which is the sum of the CV's associated with measurement of nuclear size, shape, and texture. It is remarkable that in a number of studies, alteration of nuclear shape alone has proven to be a better predictor of mortality in stage A2 prostatic cancer than has the Gleason, Mostofi, or Johns Hopkins grading systems, or ploidy status[22~24]. Multivariate analysis of up to 16 nuclear shape descriptors, including nuclear roundness factor, variance of roundness factor, and nuclear ellipticity, have accurately predicted recurrence of cancer after surgery in 11 of 26 patients with renal cell carcinoma[25], 7/14 patients with transitional cell carcinoma of the bladder[26], and 17/27 patients with Wilms' tumor (kidney)[27].

4) Nucleolar morphometry (number, size, shape, position, and pleomorphism)

Nucleoli are ribosome factories expressed by genes located on the acrocentric chromosomes 13, 14, 15, 21, and 22. In the activation of proliferation or secretion, the size and sometimes the number of nucleoli increases. In a study of eight nuclear and nine nucleolar morphometric features in breast cancer, simple nucleolar frequency, or the total number of nucleoli per 100 nuclei, was the best single predictor of recurrence-free survival[28]. Changes in nucleolar morphometry have been reported to be a correlate of the extent of neoplastic progression in prostatic intraepithelial neoplasia[29,30]. Using the silver stain for nucleolar organizing regions, the AgNOR stain, in lesions of the colorectum, the mean number of nucleolar organizer regions (NORs) clearly distinguish between tubular adenomas, villous adenomas with moderate nuclear atypia, villous adenomas with severe nuclear atypia, and colorectal adenocarcinoma[31].

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