Journal of Cellular Biochemistry 17G:37–43 (1993)

Strategies for the Application of Biomarkers for Risk Assessment and Efficacy in Breast Cancer Chemoprevention Trials

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Abstract Current chemoprevention trial designs based on epidemiological risk assessment and occurrence of cancer as an endpoint are inefficient and expensive. Novel biomarkers are needed to facilitate the development of chemopreventive interventions. The following four categories of biomarkers may be useful in prevention trials: histologic and morphometric markers; phenotypic markers of dysregulated proliferation, differentiation, and cell loss; specific oncogenes and growth regulators which are qualitatively or quantitatively altered in breast cancers; and markers of genetic and epigenetic instability. Some of these markers will be generally useful regardless of the chemopreventive approach used, whereas others may be uniquely useful in trials of specific chemopreventive agents [*e.g.*, upregulation of progesterone receptor (PR) expression in response to tamoxifen]. The development of these markers requires three phases of study: "Phase I": assessing the prevalence of the putative marker in malignant and premalignant tissue from individuals who have developed breast cancer; "Phase II": assessing *in vivo* modulation of the biomarker by the proposed chemopreventive agent; and "Phase III": applying the proposed biomarker in larger-scale trials of chemopreventive agent in high-risk populations, either before or after the development of a primary breast malignancy. The use of these biomarkers may also allow identification of novel targets for chemoprevention.

Key words: Breast cancer, chemoprevention, genetic instability, intermediate biomarkers, multistep carcinogenesis

Developing chemopreventive strategies for breast cancer requires accurate means to identify women at high risk for development of breast cancer; an available, potentially effective preventive intervention; and well-designed clinical trials to determine the efficacy of the proposed intervention. Risk assessment is routinely performed based on epidemiological and biological lifestyle factors [1,2]. However, there are serious limitations to this approach. Three-fourths of all breast cancers occur in women with no identifiable epidemiological risk factors [3]. Conversely, there is virtually no identifiable group among high-risk women with a greater than 30% lifetime risk of breast cancer (an exception is a relatively small number of families with hereditary breast cancer in whom the lifetime risk for first-degree

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relatives may approach 50%). Therefore, chemopreventive interventions have to be applied to a large number of women, the majority of whom are not destined to develop breast cancer. For example, in the Breast Cancer Prevention Trial being conducted by the National Surgical Adjuvant Breast and Bowel Project, approximately 8,000 women will receive tamoxifen for an expected absolute reduction of about 60 cases of breast cancer. Even if the highest risk individuals could be identified, their risk of breast cancer development would extend throughout their lifetime; the chemoprevention trial would have *to be long if cancer* is the endpoint. This makes the execution of chemoprevention trials very expensive and limits the number of chemopreventive agents that can be tested. One way to overcome these drawbacks is to develop novel markers to identify high-risk women in the general population as well as those who are at the highest risk for breast cancer among the various epidemiologically defined high-risk groups. In addition, markers need to be identified that can be useful as surrogate, interim endpoints of efficacy in chemoprevention trials (*i.e.*, intermediate biomarkers) to supplement cancer as a therapeutic endpoint.

POTENTIAL BIOMARKERS FOR BREAST CANCER CHEMOPREVENTION TRIALS

Potential biomarkers for breast cancer chemoprevention trials may be divided into the following four broad categories: (a) histologic and morphometric markers, (b) proliferation, differentiation, and invasion markers, (c) specific oncogenes/growth regulators, and (d) markers of genetic and epigenetic instability.

Histologic and Morphometric Markers

Histologic evidence of increased cellular proliferation and basement membrane invasion remains the *sine qua non* of malignancy. However, there is no consensus as to the nature and morphology of premalignant breast lesions. The presence of atypical proliferative lesions or lobular *in situ* carcinoma correlates with a higher subsequent risk of breast cancer [2]; however, almost half of these cancers develop in the contralateral breast. This would suggest that these lesions identify a tissue field at risk for developing malignancy rather than serve as precursors of malignancy themselves [4]. Another problem with histologic markers is that only a small proportion of women undergo breast biopsy during their lifetime; of those who do, less than 5% will have atypical hyperplasia. In addition, the diagnosis of these lesions requires at least a core biopsy. Repeated performance of such an invasive procedure during the course of a prevention trial is relatively impractical. The use of fine needle aspirates, especially in conjunction with image cytometric analysis, may provide a less invasive means for evaluating these lesions [5,6]. However, there is a lack of clearly defined and uniformly accepted criteria for diagnosing atypia in fine needle aspirates [7]. Therefore, other types of markers are required that can be used to decipher the underlying changes associated with increased risk of cancer.

Proliferation, Differentiation, and Invasion Markers

Recent advances in immunohistochemical techniques have provided markers to assess the phenotypic attributes of malignant transformation, *vis-à-vis* dysregulated cellular proliferation, lack of differentiation and cell loss, and basement membrane invasion. The proliferative fraction in a cell population may be estimated by assessing the fraction of cells that stain positively for proliferating cell nuclear antigen (PCNA) or Ki-67. These markers may provide a more reliable index of proliferative activity than the mitotic index. Evidence for the undifferentiated phenotype may be seen in the expression of tumor-associated antigens (*e.g.*, CEA and CA15-3). The propensity to invade basement membrane may be indicated by the expression of proteases (*e.g.*, collagenase type IV, heparinase, urokinase-type plasminogen activator). Although invasive malignancy shows all of these features, the detection of one or more of these in the breast epithelium before histologic evidence of malignancy could be a useful biomarker.

Specific Oncogenes/Growth Regulators

One approach to identify potentially useful biomarkers is to study the specific molecular and genetic changes that occur in breast cancers and then investigate their presence in early lesions.

TABLE I. Expression of Growth Regulators in Normal and Histologically Abnormal Tissue (% Cases Examined) [23,24,27–36]

Growth Regulator	Normal/Benign Hyperproliferation	Atypical Proliferation	DCIS	Invasive Carcinoma
Tyrosine kinases				
HER-2	0	0	Comedo 70% Non-comedo 15%	15–45
EGFR	100	?	?	26–80
Nuclear proteins				
myc	64–100	?	100	100
p53	0	?	30–50	30–53
Rb loss	0	?	?	10–40
ER	34–61	?	60–90	65–90
PR	70	?	–	55
Other growth regulators				
PDGF-B	?	?	?	55
int-2	0	?	?	15 (amplification)
EGF	?	?	?	80
bFGF	100 (myoepithelial)	?	?	100 (myoepithelial)
Others				
TGF-$\beta_{1,2,3}$	~100	?	~100	~100
*nm*23	–	?	58	31

The expression of several oncogenes and growth factors has been shown to be altered in breast cancer (Table I). In recent years, considerable data have accumulated regarding the relative expression of these oncogenes and growth factors in normal breast epithelium, *in situ* carcinoma, and invasive carcinoma; the majority have been shown to occur at the stage of *in situ* carcinoma or later (Table I). Therefore, these markers may be useful in detecting the transition to *in situ* carcinoma in high-risk women, especially in individuals with suspicious histopathological changes or in those who undergo a directed biopsy. However, the utility of these as markers of breast cancer risk or chemopreventive efficacy in a random biopsy of breast tissue in a high-risk woman is unknown. An alternative approach for risk assessment that may not require directed biopsies is to develop markers that reflect the underlying processes involved in producing these end results.

Markers of Genetic and Epigenetic Instability

A large number of chromosomal abnormalities, both numerical and structural, are frequently present in breast tumors. Within a given tumor, the chromosomal complement may vary considerably from one cell to another (tumor heterogeneity). Such diversity may superficially appear to pose insurmountable obstacles to the development of biomarkers that could be applied to large cohorts of women. However, these problems may be circumvented by identifying markers of processes that underlie the evolution of such heterogeneity. For example, individuals who are heterozygotes for ataxia telangiectasia,

a condition known to be associated with increased chromosomal instability [8,9], are estimated to constitute 8–20% of all women with breast cancer in the United States [10]. Similarly, p53 mutations, which are known to confer karyotypic instability [11], are frequently found in breast cancers [12]. These abnormalities may allow mistakes to be made at diverse genetic loci during genomic replication and cell division, leading to the widespread heterogeneity observed in breast cancers. The presence of a field predisposition to breast cancer is consistent with the existence of underlying genetic instability. Manifestations of such genetic instability may be observed in preneoplastic tissue as aneuploidy (detected by image cytometry) or as random gains and losses of chromosomes (detected by chromosome *in situ* hybridization techniques). Using these techniques, we have demonstrated the presence of chromosome 17 polysomy in histologically normal and benign proliferative tissue from women with breast cancer [13]. The advantage of using such markers is that they may be present throughout the mammary epithelium and, therefore, applicable to blind biopsies. Furthermore, the frequency of chromosomal gains and losses may allow an insight into the degree of genetic change that has already gone on in the tissue at risk. At the gene level, instability may manifest itself as loss of heterozygosity (LOH) at multiple, random loci. Some of these losses may be directly related to tumorigenesis (*e.g.*, LOH at p53 locus), whereas the functional consequences of others are as yet unknown. Novel forms of instability involving somatic deletions in poly (dA.dT) sequences and other simple repeats [*e.g.*, (CA)n] have recently been described in colon cancer [14,15] and will be worthy of study in breast tumors. Other processes that may be related to genetic instability include nucleotide structure modifications [16] and abnormal methylation patterns [17]. Such changes have been shown to be associated with and precede the structural chromosomal changes in some tumors [18,19].

In a broader sense, the notion of instability may also be extended to epigenetic phenomena. Alternative splicing of mRNA molecules may allow cells to escape growth control mechanisms. For example, variant estrogen receptors (ERs) that lack exon 5 are unable to bind the ligand but have constitutive transcriptional activity [20], and may allow a cell to become independent from regulation by estrogens or antiestrogens. Similarly, there may be randomly manifest errors of post-translational modification of proteins, *e.g.*, aberrant glycosylation of cell surface receptors or cytoplasmic proteins involved in signal transduction. Occurrence of these changes in a random fashion in the epithelium at risk may provide a growth advantage to the cells by interfering with regulatory mechanisms, and genetic damage may follow as a consequence of excessive, unregulated cell proliferation. The cumulative effect of successive aberrations, epigenetic and genetic, may be the neoplastic phenotype. Therefore, detection of one or more of these in even a small percentage of cells in a high-risk individual may indicate an ongoing instability and thus be a useful biomarker of risk. Examples of abnormally glycosylated proteins in breast cancer include several blood group-related antigens, such as Lewis-b antigen, Thomsen-Friedenreich antigen, and mucin-associated antigens (*e.g.*, sialyl-Tn antigens). In colonic tissue, some of these antigens can be detected several years before the development of cancer [21]. Similar studies are also in progress in breast cancers. As the functional consequences of such epigenetic changes are identified, they may also provide novel targets for chemopreventive intervention.

CRITERIA FOR SELECTION OF BIOMARKERS

The following five parameters need to be assessed at an early stage in the consideration of a biomarker for use in chemoprevention trials.

Sensitivity

The marked heterogeneity of breast cancer suggests that markers specific to one pathway of breast tumor development may have very low sensitivity for breast cancer in general. For example, HER-2 amplification is detected in more than 70% of comedocarcinomas (which are less common) but in only 10–15% of other more common breast cancers [22]. Thus, HER-2 amplification is likely to be an insensitive marker of malignant transformation in breast epithelium in a general population of women. In contrast, markers of end results of these processes, *e.g.*, dysregulated proliferation, may be more generally applicable

and be very sensitive for detection of high-risk women.

Specificity

Application of chemopreventive intervention in a cost-effective fashion and with minimum morbidity requires that the markers used for identifying high-risk women be associated with an extremely high risk of malignancy. As an example, when the amplification of HER-2 is detected (a finding which has not been reported in normal or benign proliferative breast epithelium), it could be a very specific marker of neoplastic transformation in breast epithelium. Of course, the ideal marker would be one whose presence is associated with a 100% risk of developing breast cancer and whose absence is associated with a 0% risk of breast cancer. Given the heterogeneity of breast cancer, it is likely that multiple markers will be employed in chemoprevention trials for the foreseeable future.

Quantifiability

It is important that the proposed markers be quantifiable so as to allow their correlation with, and integration into, quantitative risk models. Quantifiable markers can also be studied serially to assess their modulation during chemopreventive intervention. Quantitation of the extent of marker aberrancy in premalignant tissue may also allow an estimate of the time frame in which a malignancy may be expected to develop in the absence of chemopreventive intervention. Markers that can only be assessed as present or absent (*e.g.*, a mutation involving a specific gene) may still be useful for risk assessment, but are likely to be of limited use for follow-up during the course of a prevention trial.

Generalized Versus Focal Expression

In the absence of readily visualizable preneoplastic lesions which could be biopsied to study expression of specific markers, the tissue material available in the context of a chemoprevention trial is likely to be a random sample of mammary epithelium. Therefore, it is important to know if the distribution of altered expression of a marker in the field at risk is global or focal. A clonal genetic abnormality that leads to tumor development may be seen only in the transformed epithelium (thus requiring a directed biopsy), whereas the underlying process (*e.g.*, genetic instability) may be manifest in the entire mammary epithelium and could be studied in a random biopsy or perhaps even in other cell types (*e.g.*, lymphocytes or fibroblasts).

Technical Considerations

In general, the markers proposed should be evaluable in readily accessible tissue (*e.g.*, blood) or in small amounts (*e.g.*, fine needle aspiration specimens) of mammary tissue. Furthermore, the results should be easily reproducible, with minimal inter-experimental variation to allow longitudinal follow-up. It is important to establish a baseline for the expression of any given marker in normal risk epithelium. A special consideration is that mammary epithelium is hormonally responsive and its physiology is cyclical. Therefore, the expression of proposed biomarkers should be analyzed in the context of the hormonal milieu at the time of biopsy. For example, the expression of ER in the normal mammary epithelium is much higher during the first half of the menstrual cycle as compared to the second half [23]. Similarly, ER expression in normal mammary epithelium in post-menopausal women is much higher than in premenopausal women [24].

APPLICATION OF BIOMARKERS IN CHEMOPREVENTION TRIALS

The utility of a potential biomarker for chemoprevention trials may be explored in three phases. In the first phase, the prevalence of the putative marker may be compared in malignant and nonmalignant breast tissue from the same individual. The nonmalignant tissue may be normal breast tissue adjacent to a tumor, the contralateral uninvolved breast, and where available, previous benign breast biopsy specimens from the same individuals. Markers that are differentially expressed in neoplastic and non-neoplastic tissue, especially those that appear to be expressed early in the neoplastic transformation, could be potentially useful for chemoprevention trials. Markers that are expressed in benign breast biopsies from women who subsequently developed breast cancer may also be potentially useful biomarkers. This phase of development of

biomarkers could be accomplished largely in retrospective studies using archival material. Laboratory studies during this phase should also include *in vitro* studies of the effect of the chemopreventive agent on various growth regulators to identify potential mechanisms of intervention. The results of these studies should be analyzed to assess the suitability of the proposed marker based on the biomarker selection criteria described earlier.

The second phase in the application of a biomarker should be its modulability by the proposed chemopreventive intervention. This could be accomplished by short-term administration of the chemopreventive agent. The duration of such a study protocol would depend on the expected time course of marker modulation by the treatment. For example, a 1–2 week period of treatment may be adequate to assess upregulation of ER, progesterone receptor (PR) [25,26], or HER-2 if tamoxifen was the proposed chemopreventive agent. In contrast, reversal of ductal carcinoma *in situ* (DCIS) or disappearance of aneuploid cells may require a treatment duration of several months. Trials of very short duration (1–2 weeks) may be carried out prior to definitive surgery in women with newly diagnosed breast cancer. Trials requiring longer-term treatment may be carried out in high-risk women, either those who are in epidemiologically high-risk groups but have not yet developed breast cancer or those who are at risk for a second primary breast cancer following potentially curative therapy for a primary breast cancer.

Markers that appear promising by virtue of their expression during malignant transformation and their downregulation by the proposed preventive intervention can then be tested in Phase III trials (preferably randomized) to validate them against the definitive endpoint (*i.e.*, reduction in cancer incidence). During this phase, the frequency and degree of downregulation of the marker should be correlated with proportional cancer risk reduction. Additional studies in this phase should include detailed biological and molecular analyses of the tumors that do develop in order to understand the mechanisms of the chemopreventive strategy's failure, and to develop means to circumvent this failure.

In conclusion, the emerging possibilities of breast cancer chemoprevention have necessitated the development of efficient trial designs incorporating surrogate endpoints of efficacy. A systematic dissection of genetic and phenotypic changes accompanying the evolution of breast neoplasms and the underlying processes responsible for these changes should allow the identification and application of novel biomarkers for prevention trials. Furthermore, these studies may also allow identification of novel targets for chemopreventive intervention.

ACKNOWLEDGMENTS

Kapil Dhingra is a recipient of a Clinical Oncology Career Development Award from the American Cancer Society. We wish to thank Judy Vance for preparation of the manuscript.

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