Journal of Cellular Biochemistry Supplements 28/29:101–110 (1997)

Breast Cytology and Biomarkers Obtained by Random Fine Needle Aspiration:Use in Risk Assessment and Early Chemoprevention Trials

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Abstract In a prospective pilot study, we performed breast fine needle aspirations (FNAs) on 224 high-risk and 30 low-risk women and analyzed these aspirates for cytologic changes and biomarker abnormalities of aneuploidy and overexpressed estrogen receptor (ER), epidermal growth factor receptor (EGFR), p53 and HER-2/*neu*. High-risk women had a first-degree relative with breast cancer (74%), prior biopsy indicating premalignant breast disease (25%), a history of breast cancer (13%), or some multiple of these risk factors (12%). Median ages of the high- and low-risk groups were 44 and 42, respectively. Seventy percent of high-risk and 17% of low-risk women had cytologic evidence of hyperplasia with or without atypia ($P < .0001$). Aneuploidy and overexpression of EGFR and p53 occurred in 27, 37, and 29% of high-risk subjects but only 0, 3, and 3% of low-risk subjects ($P < .0023$). Overexpression of ER and HER-2/*neu* occurred in 7 and 20% of high-risk women but in none of the low-risk subjects. Biomarker abnormalities were more frequent with increasing cytologic abnormality. Restricting the analysis to those 3 biomarkers most frequently overexpressed in the high-risk group (ploidy, EGFR, p53), 13% of high-risk women with normal cytology, 19% of high-risk women with epithelial hyperplasia, and 49% of high-risk women with hyperplasia with atypia had abnormalities of 2 or more of these 3 biomarkers ($P = .00004$). At a median follow-up of 32 months, four women have been diagnosed with invasive cancer and two with ductal carcinoma in situ (DCIS). Later detection of these neoplastic conditions was associated ($P \leq .016$) by univariate analysis with prior FNA evidence of hyperplasia with atypia; overexpression of p53 and EGFR; the modified Gail risk of breast cancer development at 10 years; and multiple biomarker abnormalities. By multivariate analysis, later detection of cancer was primarily predicted by the number of biomarker abnormalities in the 3-test battery ($P = .0005$) and secondarily by the Gail risk at 10 years ($P = .0049$). In turn, hyperplasia with atypia was associated with multiple biomarker abnormalities, particularly p53 and EGFR overexpression. Thus, hyperplasia with atypia and cytologic markers in breast FNAs have promise as risk predictors and as surrogate endpoint biomarkers for breast cancer chemoprevention trials. J. Cell. Biochem. Suppls. 28/29:101–110.

Key words: biomarker; breast; breast cancer development; chemoprevention; clinical trials; cytology; ER; EGFR; fine needle aspiration; FNA; HER-2/*neu*; high risk; p53; ploidy; risk assessment; surrogate endpoint

Most clinical evidence for chemoprevention in breast cancer has been derived from large randomized trials of women with Stage I and II disease undergoing adjuvant treatment with tamoxifen or fenretinide (4-HPR), in which the endpoint is a decrease in the prevalence of a new contralateral tumor [1–4]. Prospective, randomized trials of high-risk women without prior invasive cancer are currently ongoing. However, the cost of the U.S. National Surgical Adjuvant Breast and Bowel Project trial of tamoxifen vs. placebo in high-risk women has been estimated at over $60 million [5]. Entry of over 13,000 participants has required 5 years; follow-up of another 5 years or more will be required to fully study possible therapeutic effects and toxicity.

In order to efficiently evaluate the large number of compounds with potential chemopreventive activity currently available, new models are needed which will evaluate these agents by studying changes induced at the tissue level, before large scale Phase III trials are per-

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Received 6 January 1997; Accepted 10 November 1997

formed [6]. Issues yet to be resolved in breast Phase II chemoprevention trials include identification of the most appropriate cohort and optimum tissue biomarkers for testing, tissue sampling methods, length of study, confidentiality, and appropriate reimbursement for direct trial expenses and for time lost from work or other activities.

The most appropriate cohorts for Phase II trials are those who consider themselves at short-term high risk for developing breast cancer and who possess the appropriate breast tissue biomarkers. Women with histologic evidence of atypical intraductal hyperplasia (AIDH), lobular carcinoma in situ (LCIS), and ductal carcinoma in situ (DCIS) all have a marked increased short-term risk for invasive breast cancer and also possess the requisite tissue biomarker. US women with AIDH and DCIS will generally have the index lesion removed at the time of discovery. Women with DCIS will often undergo mastectomy or breast radiation, as well.

Twenty-five thousand cases of DCIS are discovered annually in the US [7]. Since DCIS is most often discovered on screening mammography and is not palpable, stereo tactic needle biopsy is often the method of diagnosis. Theoretically, residual DCIS often remains behind until re-excision or mastectomy. Chemopreventive agents could be evaluated in this interval.

Three ongoing Phase II trials in the US are studying incompletely ressected DCIS in women. Women are randomized to receive drug or placebo in a double-blind fashion for 2–4 weeks. Tissue obtained before and after treatment is studied for global morphologic and grade changes, nuclear morphometric changes (nuclear and nucleolar area, chromatin pattern, etc.), ploidy, proliferation indices, and tumor suppressor and oncogene expression [8]. With this approach, women are not subjected to extra invasive procedures and a substantial amount of tissue is obtained for study. However, problems include short treatment time, limited number of potential participants and low trial acceptance rate, tissue heterogeneity, and removal of the entire DCIS lesion with the larger bore (14 and 11 gauge) stereotactic needles. In the initial 12 months after opening, accrual to all three US DCIS trials was low.

An alternate approach is the use of random fine needle aspiration (FNA) to detect cytologic and molecular abnormalities in high-risk women. This approach might permit a much larger cohort of participants, an earlier intervention in the neoplastic process, prolonged drug administration, and repeated tissue sampling by a minimally invasive procedure. A critical question is whether morphologic and molecular abnormalities are sufficiently widespread throughout the breast tissue to allow detection by random sampling, and whether these abnormalities, if detected, would be predictive of short interval cancer development. Important leads were provided by Marshall et al. [9] who found evidence of moderate to severe hyperplasia with or without atypia in 39% of 51 women who had a first-degree relative with breast cancer, using random four quadrant FNA. Using nipple aspirate cytology, Wrensch et al. found that women with atypia in their nipple aspirates developed breast cancer with greater frequency than those with normal or acellular aspirates [10].

We hypothesized that a number of molecular markers reflective of a dysregulated or hyperproliferative state would become more prevalent during the promotion and progression phases of neoplastic development, and that these abnormalities might be detected using random FNA and immunocytochemical staining techniques. We reasoned that if a set of morphologic and molecular markers could predict which high-risk women would develop breast cancer in a 5-year time interval, FNA methodology could be used to select an ideal cohort for Phase II chemoprevention trials.

METHODS

High-risk women were defined as having one or more of the following risk factors: a first-degree relative with breast cancer; a prior breast biopsy interpreted as showing evidence of atypical intraductal hyperplasia (AIDH) or carcinoma in situ; or node negative breast cancer. High-risk women, self-referred or referred by a variety of community and regional physicians, were generally between the ages of 30 and 60. Women older than 60 were not aspirated in the absence of clinical evidence of moderate to severe proliferative breast disease because of difficulty obtaining adequate cells from the involutional breast. Women younger than 30 were aspirated only if they were within 10 years of the age at which their youngest first- or second-degree relative developed breast cancer.

Low-risk women were defined as those with none of the above major risk factors. In addi-

tion, low-risk women were between the ages of 30 and 60, had their first live birth before age 30, had no clinical evidence of proliferative breast disease and no first- or second-degree relatives with breast or ovarian cancer. Low-risk women were paid volunteers recruited through an ad in the medical center newspaper. A modified Gail-estimated probability of breast cancer development at 10, 20, and 30 years from entry was calculated for each woman using age at menarche, age at first live birth, current age, number of first-degree relatives with breast cancer, number of breast biopsies, and prior biopsy showing atypia or cancer as variables [11]. However, the Gail risk estimate was not used to determine eligibility.

All women were required to have a mammogram performed within 12 months prior to aspiration and interpreted as not suspicious for breast cancer. Clinical breast exam just prior to the aspiration must also have been interpreted as not suspicious for breast cancer. Estrogen replacement therapy was permitted.

Procedures for aspiration, tissue processing, cytologic criteria, and assay analysis have been previously detailed but will be reviewed here [12–15].

After alcohol and betadine cleansing, local skin anesthetic was placed with a tuberculin needle just lateral to the areola at approximately 3 and 9 o'clock (Fig. 1). The position might be altered to avoid blood vessels and prior surgical scars. An additional 2–5 cc of local anesthetic was injected deep into the breast tissue. The anesthetic was a mixture of 1 part bicarbonate and 3 parts 1% lidocaine with epinephrine. Bicarbonate was used to decrease discomfort and cellular distortion by maintaining pH in a physiologic range. A 1–1/2" 21 gauge needle attached to a 12 cc plastic syringe prewet with ice cold sterile RPMI 1640 tissue culture media was used to perform the aspiration. The needle was positioned just lateral to the areola at a 30–80° angle to the chest wall (Fig. 1). Four to 10 needle passes within the breast parenchyma were performed before the needle was withdrawn. The procedure was repeated with 4–5 needle-syringe set-ups on each side of the breast. The procedure was stopped if moderate to marked bleeding was encountered.

Laboratory personnel were present in the clinic at the time of aspiration to collect cells immediately. Cells from both breasts were pooled by expelling them into a 5 cc tube of ice cold tissue culture media. The tube was kept in an ice bath until the cells were processed further (within 1–2 hours). Following the aspiration, cold packs were applied to the breasts for 10 minutes. The breasts were bound for approximately 18 hours and women were instructed to wear a spandex sports bra for several days and avoid aspirin and non-steroidal anti-inflammatory drugs. Each woman was initially aspirated twice, 6 months apart. The pooled results from these two aspirates were used as the "initial aspirate." Women with hyperplasia with atypia were then reaspirated yearly. All other women were reaspirated every 2–3 years depending on the number of abnormal biomarker tests. Women having FNA evidence of hyperplasia with atypia were encouraged to undergo a clinical breast exam 3–4 times yearly and twice-yearly mammography. Others were encouraged to continue with yearly clinical breast exam and mammography.

Approximately 1/3 of the aspirate was aliquoted for cytology and the remaining 2/3 for the other biomarker tests. Thus, there was always twice as much material aliquoted for cytology as for each of the other tests. A red cell lysing buffer consisting of 0.1 M ammonium nitrate and EDTA was applied to the specimen. Cells for cytology were filtered (not smeared) through a 25 mm millipore filter with a 5 μm membrane. This procedure decreased cellular loss and morphologic distortion. For the past 2 years, 1 cc of a 0.25 mg/ml collagenase solution has been applied to the aliquot for ploidy and biomarker assessments. This procedure dispersed the cells into a monolayer, necessary for nuclear morphometry studies. The collagenase step did not appear to decrease the prevalence of biomarker positivity but did decrease the prevalence of aneuploidy.

One pathologist (CZ) interpreted all slides. Periodically, sets of 25 slides were re-evaluated with the pathologist blinded to results of prior interpretations. Intraobserver variance was 8% (8/100). In addition, the 25-slide set was interpreted by an additional pathologist using the same criteria. Interobserver variance was 14% (10/75).

Sampling variance was estimated by analyzing differences in cytologic classification in 43 consecutive subjects in whom aspirations were performed at 6-month intervals. Eleven subjects had hyperplasia with atypia (AH) in their first aspirate. Six of the eleven (60%) had AH in

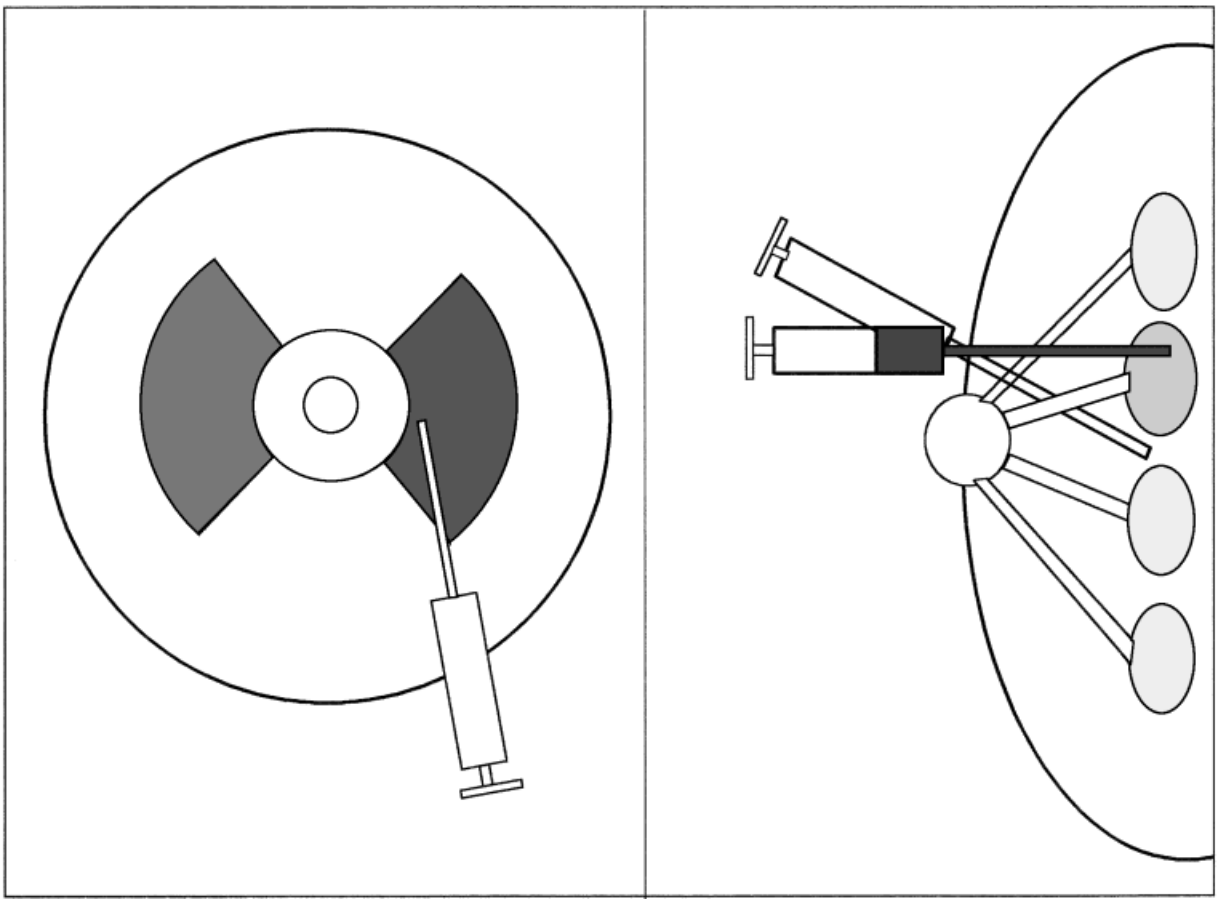

Fig. 1. Schematic of the breast aspiration procedure. Areas adjacent and lateral to the areola are probed to aspirate fluids and cells from the underlying ductal regions.

their second aspirate and 30% had epithelial hyperplasia without atypia (EH) in their second aspirate. Nineteen subjects had EH in their first aspirate. Nine of nineteen (53%) had EH in their second aspirate and 21% had AH in their second aspirate. Thus, consistency was 76% for proliferative cytology (EH or AH) between the first and second aspirate. The quantity-not-sufficient rating by cytologic category for any biomarker test was 6% for AH, 16% for EH, and 30% for women with normal, non-proliferative cytology.

A standard Feulgen's procedure was used to determine ploidy with analysis on a CAS-200 system. One hundred cells were counted. A DNA index of ≤ 0.85 or >1.15 was considered aneuploid.

Cells for estrogen receptor (ER) and epidermal growth factor (EGFR) were fixed in 10% buffered formalin for 7 minutes, absolute −20°C methanol for 3 minutes, and −20°C acetone for 1 minute. ER was determined with reagents and procedures in the Abott (Abbott Park, IL) ER-ICA kit. EGFR was determined using clone F4 anti-EGFR antibody (Sigma, St. Louis, MO). Cells for both p53 and HER-2/*neu* were fixed in acetone and assayed using Pab240 (p53) and Ab#3 (HER-2/*neu*) both from Oncogene Science (Uniondale, NY) [15]. Cells were scored from 0–3+ separately by each of two reviewers (SK, SZ). Slides with 2–3+ cell clumps were considered as positive or antigen overexpression. The two reviewers independently interpreted all immunocytochemistry slides. Intraobserver variance was 4%.

Statistical analysis was performed using SPSS for Windows (Release 6.1 SPSS, Inc., Chicago, IL). Standard methodologies were used to calculate *P* values [16]. Multiple regression equations were constructed for cytologic categories and each of the other biomarkers [17,18]. Modifying variables for cytologic categories included the other biomarkers, menopause status, current age, modified Gail risk estimate,

and risk factor subcategory. When the individual biomarkers were used as the dependent variable, cytologic designation was included as the modifying variable.

Eventual development of either DCIS or invasive lobular or ductal cancer was also analyzed by multiple regression. Modifying variables included cytologic category, biomarker results, 10- and 30-year modified Gail risk estimates, menopause status, and age. Also performed were regressions using presence or absence of multiple abnormalities in the five-test set (ploidy, ER, EGFR, p53, and HER-2/*neu*) or the three-test set (ploidy, EGFR, and p53), instead of the individual biomarkers as modifying variables.

RESULTS

The present analysis is limited to 224 high-risk women and 30 low-risk women entered between March 1991 and May 1996, for whom cytology and five biomarker tests were attempted and/or completed. Comparisons of demographic variables for high- and low-risk groups are shown in Table I. The median age of the high-risk group was 44 (age range 29–65); median age in the low-risk group was 42 (31–52); the majority of women were premenopausal. Thirty-eight percent of the high-risk group vs. none of the low-risk group had either never had children or had their first child after 30. Twenty-eight percent of high-risk women vs. 17% of low-risk women had either previously received or at the time of entry into the study were receiving estrogen replacement therapy.

TABLE I. Demographic Factor Distribution in Low- and High-Risk Groups

	High-risk (n = 224)	Low-risk (n = 30)	*P* value
Median	44	42	0.0022
Age range	31–52	29–65	
% >50	25%	7%	0.022
No live birth < age 30	38%	0%	0.00003
% Premenopausal	65%	73%	—
Estrogen at entry	12%	10%	—
Replacement (previous)	16%	7%	—
Median 10-year Gail risk	5%	1%	0.0001
Median 30-year Gail risk	15%	5%	0.0001

Seventy-four percent of high-risk women had at least one first-degree relative with breast cancer. Seventeen percent of high-risk women had four or more relatives with breast cancer and thus may belong to a hereditary cancer family [19]. Twenty-five percent of the high-risk women had a biopsy indicating prior precancerous disease (AIDH, DCIS, LCIS), 13% had prior breast cancer, and 12% had a combination of the above three risk factors. The median 10- and 30-year Gail probability of breast cancer development in the high-risk group was 5 and 3 times, respectively, that of the low-risk group (Fig. 2).

Cytology Patterns in the High- and Low-Risk Groups

The prevalence of normal, non-proliferative cytology was only 30% in high-risk women, as compared to 83% in the low-risk group. Fifty-one percent of high-risk but only 17% of low-risk women had evidence of EH in their FNA. Nineteen percent of high-risk and no low-risk women had evidence of AH in their FNA. Differences in the prevalence of normal, hyperplastic, and atypical FNA cytology patterns between high- and low-risk women were statistically significant ($P < 0.0001$).

Prevalence of Biomarker Expression in the High- and Low-Risk Groups

The prevalence of biomarker expression in the high- and low-risk groups is shown in Table II. EGFR and p53 were the most frequently overexpressed markers in the high-risk group, with prevalence rates of 37 and 29%, respectively. Except for ER, differences in the prevalence of ploidy abnormalities or biomarker overexpression between high- and low-risk groups were all statistically significant. If a 2+ classification of antibody-labeling was the cutoff for ER overexpression, then it occurred in only 7% of high-risk women. Horsfall et al. [20] have shown that ER staining intensity is often reduced in cytospin preparations. If preparations with 1+ classification are included as overexpressed, the prevalence of ER overexpression doubles (15%) and the difference between high- and low-risk women approaches statistical significance ($P = .077$).

Seventy percent of high-risk women, but only 7% of low-risk women, exhibited one or more biomarker abnormalities. Thirty-one percent of

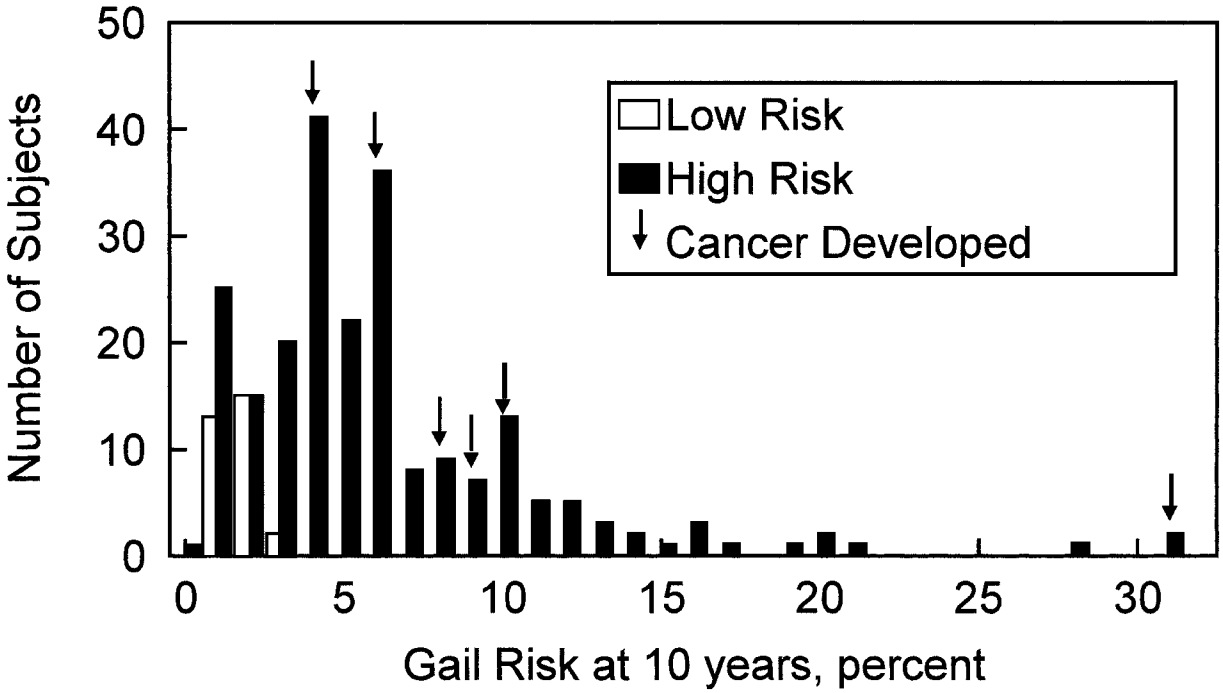

Fig. 2. Distribution of projected probabilities of breast cancer development within 10 years (Gail Risk) for high- and low-risk women. Also shown are the individual values exhibited by six women who subsequently developed DCIS or invasive breast cancer.

TABLE II. Distribution of Biomarker Abnormalities in Low- and High-Risk Groups

Biomarker	High-risk (n = 224) (%)	Low-risk (n = 30) (%)	*P* value
EGFR overexpression	37	3	0.0001
p53 overexpression	29	3	0.0023
DNA aneuploidy	25	0	0.0033
HER-2/*neu* overexpression	20	0	0.011
ER overexpression (≥2+)	7	0	0.20
ER overexpression (≥1+)	15	3	0.077
≥1 Abnormality (5-set)	70	7	<0.00001
≥1 Abnormality (3-set)	64	7	<0.00001
≥2 Abnormalities (5-set)	31	0	0.00032
≥2 Abnormalities (3-set)	23	0	0.0031

the high-risk women had two or more biomarker abnormalities, none of the low-risk women did. If the analysis is restricted to the three-biomarker panel of ploidy, p53, and EGFR, 64% of high-risk women had at least a single biomarker abnormality and 23% had multiple biomarker abnormalities (Table II).

Association of Biomarker Expression With Cytologic Pattern

The prevalence of individual biomarker abnormalities in the high-risk population was significantly associated with cytologic abnormality (Fig. 3). The association was particularly striking for EGFR and p53. EGFR was overexpressed in 21% of women with normal cytology, 38% of those with EH, and 58% of those with AH in their FNAs (normal vs. EH, $P = .019$; EH vs. AH, $P = .021$; and normal vs. AH, $P = .00007$). p53 was overexpressed in 12% of women with normal FNA cytology, 29% of those with EH, and 54% of those with AH (normal vs. EH, $P = .0083$; EH vs. AH, $P = .0042$; normal vs. AH, $P = .008$). p53 and EGFR overexpression were strongly predictive of concurrent AH in the FNA ($P < .0001$). A statistically significant difference between normal cytology and AH was observed for HER-2/*neu* overexpression (10 vs. 30%; $P = .0087$), and ER overexpression approached significance (3 vs. 12%; $P = 0.070$). If ER overexpression was defined as 1+ antibody labeling instead of 2+, the distribution by cytology (normal, 9%; EH,

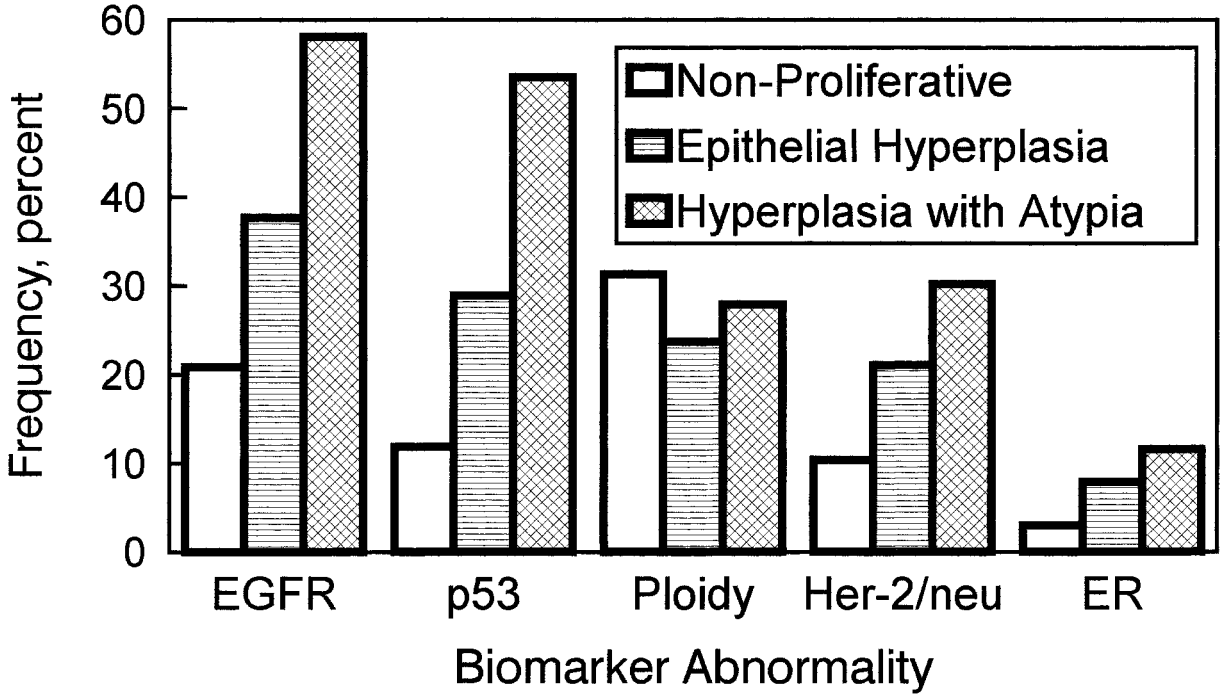

Fig. 3. Distribution of biomarker abnormalities by cytologic category of normal (non-proliferative), epithelial hyperplasia, or hyperplasia with atypia.

14%; and AH, 28%) became significant (P = .023).

The prevalence of multiple biomarker abnormalities was also associated with increasing cytologic abnormality. Using the five-biomarker panel, 16% of high-risk women with normal cytology, 30% of women with EH, and 58% of high-risk women with AH had two or more biomarker abnormalities (normal vs. EH, P = .044; EH vs. AH, P = .0011; and normal vs. AH, P = .00001). If only the three-biomarker panel is considered, 19% of women with EH and 49% of women with AH had two or more biomarker abnormalities (P = .00005). A significant correlation was found when 10- and 30-year Gail risks were compared by the absence or presence of multiple biomarker abnormalities in the three-test set (P = .0080 and .0097), but not in the five-test set, nor in cytology. Finally, premenopausal status correlated only marginally with cytology (P = .034) but not with any aspect of biomarker abnormality.

Association of Cytologic and Biomarker Abnormalities With Later Cancer Development

At a median follow-up of 32 months from the time of initial aspiration for the entire group, two of the 224 high-risk women have developed DCIS and four have developed invasive breast cancer. An additional three women have developed LCIS. Hazard function analysis indicates a projected 2.8% of women developing DCIS or invasive breast cancer at the median follow-up of 32 months. Univariate analyses (Table III) indicate that short-interval DCIS and invasive cancer development are predicted by the Gail risk at 10 or 30 years, cytologic evidence of AH, overexpression of p53 or EGFR, and the number of biomarker abnormalities or simply the presence of multiple abnormalities in the three- or five-biomarker set ($P \leq .023$). If ER overexpression was defined as 1+ antibody labeling instead of 2+, then it was also associated with cancer development (P = .016). Multivariate analysis indicates that the number of abnormal biomarkers in the three-test set (P = .0005) and Gail risk at 10 years (P = .0049) are the strongest independent predictors of short-interval cancer development (Table III).

Considering the six cases of cancer, four of the women exhibited AH, five EGFR overexpression, five p53 overexpression, four p53 and EGFR overexpression, and five multiple biomarker abnormalities in the three-test set. In contrast, only three of the six exhibited DNA aneuploidy. Use of the five-test biomarker panel did

TABLE III. Associations With Subsequent Cancer Development/Detection

Factors identified	*P* value
Univariate analysis	
Gail risk at 10 years	0.0052
Gail risk at 30 years	0.023
Epithelial hyperplasia w/o atypia	0.089
Hyperplasia with atypia	0.0028
EGFR overexpression	0.016
p53 overexpression	0.0026
DNA aneuploidy	0.19
HER-2/*neu* overexpression	0.85
ER overexpression (≥2+)	0.36
ER overexpression (≥1+)	0.016
Number of abnormalities (5-tests)	0.00001
Number of abnormalities (3-tests)	0.00009
Multiple markers (5-set)	0.0053
Multiple markers (3-set)	0.00041
Multivariate Cox Regression (in order of entry)	
1. Number of abnormalities (3-test set)	0.0005
2. Gail risk at 10 years	0.0049
3. Hormone replacement (omission does not alter model)	0.027

TABLE IV. Cytology and Biomarker Correlations With Subsequent Cancer Development/Detection*

AH* plus any combination	4/43
AH plus p53	4/23
AH plus EGFR	4/25
p53 plus any combination	5/64
EGFR plus any combination	5/82
p53 plus EGFR	4/29

	5 Biomarker set	3 Biomarker set
None or single biomarker	1/154	1/172
Multiple biomarkers	5/70	5/52
AH plus none or single biomarker	0/18	0/22
AH plus multiple biomarkers	4/25	4/21

*Six cases of cancer, 4 invasive and 2 DCIS. AH = epithelial hyperplasia with atypia.

not alter the results substantially. Five of the six women had multiple biomarker abnormalities; and ER or HER-2/*neu* overexpression was observed in one woman each. If the threshold for ER expression was lowered to 1+, then two of six women had ER overexpression, and all six exhibited multiple biomarkers in the five-test set. The positive predictive value of cytologic and biomarker combinations for later cancer development, excluding the three cases of LCIS, are listed in Table IV.

Our results show that the chance of women with neither atypia nor multiple positive markers (three-test set) developing short-interval breast cancer is only l/150, compared to l/53 for women with AH or multiple positive markers, and 4/21 for subjects with both AH and multiple biomarkers. The time-dependent development/detection of breast cancer for these three groups is graphically illustrated in Figure 4. Similar results (not shown) were obtained for the five-test set. The combination of AH plus multiple biomarker abnormalities, or AH plus EGFR or p53 overexpression are the most specific for short interval cancer development with a positive predictive value of ≥ 2% per year.

DISCUSSION

We have demonstrated that a significant portion of our cohort of predominantly premenopausal high-risk women has proliferative breast cytology by our criteria [15], and 23% display multiple biomarker abnormalities in the three-test set.

Our 32-month analysis of 224 high-risk women, which excludes LCIS as a cancerous event, shows that multiple positive markers and AH predict for later DCIS or invasive breast cancer. These findings are consistent with our previous analyses, which included LCIS as a cancerous event [14]. When LCIS is excluded, multiple biomarker abnormalities become a stronger predictor than AH and, for the first time, modified Gail risk at 10 years is a significant predictor of short-interval cancer development in the multivariate analyses. Note in Figure 2 that all but one case of cancer are associated with Gail 10-year risk above-the-median values. Multivariate analysis has previously found a consistent association between overexpression of EGFR or p53 and concomitant AH. These two variables are predictive of later cancer development/detection by univariate analysis and appear to be the two most critical biomarker assays, in addition to cytology. Aneuploidy is the third most frequently expressed biomarker in the high-risk population as a whole, as well as the subset that developed a later cancerous event. Interestingly, it is the only biomarker that shows no correlation with cytologic classification (Fig. 3). Predictive sensitivity does not seem compromised by excluding ER and HER-2/*neu* from the test panel. Since there are a significant number of acellular samples, particularly in

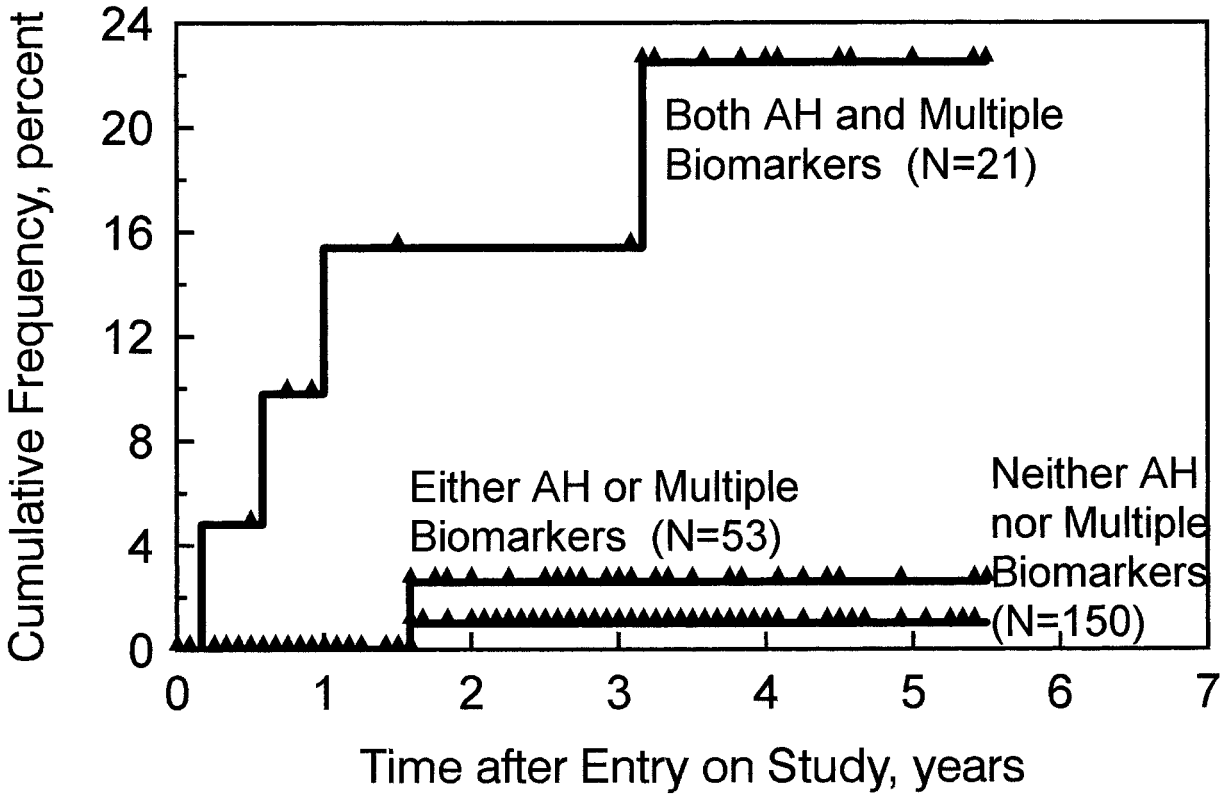

Fig. 4. Hazard function plot of the development/detection of DCIS or invasive breast cancer as a function of time after entry onto the study. The three groups consist of women with neither AH nor multiple biomarker abnormalities, women with either AH or multiple biomarker abnormalities, and women with both AH and multiple biomarker abnormalities. The triangles indicate censoring of subjects.

women with normal non-proliferative cytology, reducing the total number of specimen slides by one-third would greatly reduce the chances of insufficient cells on any one slide.

This FNA methodology is ideal for Phase II chemoprevention trials of longer term than the current DCIS trials, provided inherent limitations (e.g., tissue heterogeneity, sampling variance, difficulties in quantitation) are recognized. Women with AH or multiple positive biomarkers have sufficient short-term risk to justify the potential risk of side effects, and are also more likely to remain motivated. Cytology and the three-biomarker panel of p53, EGFR, and ploidy appear from preliminary studies to be validated as surrogate endpoint biomarkers (SEBs), although longer follow-up and more study will be required. To quantify morphology changes, the NCI chemoprevention program is requiring that nuclear morphometry be included in the biomarker panel. This is easily done on the Feulgen-stained slide for ploidy evaluation, as long as collagenase has been added to achieve a monolayer preparation.

We are currently exploring an immunofluorescent methodology which will allow us to assay more than one biomarker per cytospin slide and to better quantitate the immunocytochemistry results. Hormone receptor assays and apoptotic indicators would be ideal additions to the basic panel, especially if a hormone-like agent were being tested in a chemoprevention trial. Before ER expression can be considered useful as a SEB, cytology assay sensitivity needs to be studied further. Horsfall et al. have previously demonstrated that ER assays in cytospins have lower stain density than histologic preparations [20]. The prevalence of ER in our population seems inordinately low (7%) and this may be the reason why.

Under the auspices of the NCI Chemoprevention Branch, we have initiated a 6-month, double-blind Phase II trial of Difluoromethylornithine (DFMO) vs. placebo. Women at increased epidemiologic risk of breast cancer will be selected for possible participation in the trial by a two-tiered process in which they first undergo random FNA breast aspiration in a high-

risk clinic. Blood will be drawn and stored frozen at the time of the aspiration and the day of the menstrual cycle will be recorded. Those individuals who have atypia and/or multiple positive markers will be offered participation if they meet the other eligibility requirements. After 6 months of study drug, women will be reaspirated during the same portion of the menstrual cycle. The main study endpoint will be the cytologic change between the two groups in aspirations performed pre- and post-drug administration. Cytologic features will be scored using the semiquantitative index of Masood et al. [21]. Proliferation indices (proliferating cell nuclear antigen, PCNA), DNA ploidy and nuclear morphometry, and change in p53 and EGFR expression, will also be studied in an exploratory fashion. This trial was activated and subject entry begun in June of 1997.

ACKNOWLEDGMENTS

The secretarial assistance of Rebecca McNeely is acknowledged with gratitude, as are the efforts of Jennifer Klemp in coordinating the entire High Risk Breast Cancer program.

REFERENCES

1. Morrow M, Jordan VC (1994): The prevention of breast cancer with tamoxifen. In Jordan VC (ed): "Long-term Tamoxifen Treatment for Breast Cancer." Madison: University of Wisconsin Press, pp 257–278.
2. Boccardo F, Amoroso D, Rubagotti A, Capellini M, Pacini P, Castagnetta L, Traina A, Farris A, Iacobelli S, Mustacchi G, Nenci I, Piffanelli A, Sismondi P, De Sanctus C, Mesiti M, Gallo L, Villa E, Schieppati G (1994): Prolonged tamoxifen treatment of early breast cancer: The Experience of the Italian Cooperative Group for Chemohormonal therapy of early breast cancer. In Jordan VC (ed): "Long-term Tamoxifen Treatment for Breast Cancer." Madison: University of Wisconsin Press, pp 159–179.
3. Houghton J, Riley D, Baum M (1994): The Nolvadex Adjuvant Trial Organization (NATO) and the Cancer Research Campaign (CRC) trials of adjuvant tamoxifen therapy. In Jordan VC (ed): "Long-term Tamoxifen Treatment for Breast Cancer." Madison: University of Wisconsin Press, pp 93–113.
4. Costa A, Decensi A, DePalo A, Veronesi G (1996): Breast cancer chemoprevention with retinoids and tamoxifen. Proc Am Assoc Cancer Res 37:655.
5. Mulshine J (1995): Fostering chemopreventive agent development: How to proceed? J Cell Biochem Suppl 22:254–259.
6. Kelloff GJ, Boone CW, Steele VE, Crowell JA, Lubet R, Doody LA, Greenwald P (1993): Development of breast cancer chemopreventive drugs. J Cell Biochem 17(Suppl G):2–13.
7. O'Shaugnessy JA (1996): Chemoprevention of breast cancer. JAMA 275:1349–1353.
8. Dhingra K (1995): A phase II chemoprevention trial design to identify surrogate endpoint biomarkers in breast cancer. J Cell Biochem 23 (Suppl):19–24.
9. Marshall CJ, Schumann GB, Ward JH, Riding JM, Cannon-Albright L, Skolnick M (1991): Cytologic identification of clinically occult proliferative breast disease in women with a family history of breast cancer. Am J Clin Pathol 95:157–165.
10. Wrensch M, Petrakis NL, King EB, Lee MM, Miike R (1993): Breast cancer risk associated with abnormal cytology in nipple aspirates of breast fluid and prior history of breast biopsy. Am J Epidemiol 137:829–833.
11. Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schair C, Mulvihill JJ (1989): Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 81:1879–1886.
12. Fabian C, Zalles C, Kamel S, McKittrick R, Moore W, Zeiger S, Simon C, Kimler B, Cramer A, Garcia F, Jewell W (1993): Biomarker and cytologic abnormalities in women at high and low risk for breast cancer. J Cell Biochem 17 (Suppl G):153–160.
13. Fabian CJ, Kamel S, Kimler BF, McKittrick R (1995): Potential use of biomarkers in breast cancer risk assessment and chemoprevention trials. Breast J 1:236–242.
14. Fabian CJ, Kamel S, Zalles CM, Kimler BF (1996): Identification of a chemoprevention cohort from a population of women at high risk for breast cancer. J Cell Biochem 25S:112–122.
15. Zalles C, Kimler BF, Kamel S, McKittrick R, Fabian CJ (1995): Cytologic patterns in random aspirates from women at high and low risk for breast cancer. Breast J 1:343–349.
16. Pearson ES (1970): "Biometrika Tables for Statisticians," 3rd ed, Vol. 1. Pearson ES, Hartley HO (eds). Cambridge, MA: Cambridge University Press.
17. Dillon WR, Goldstein M (1984): "Multivariate Analysis Methods and Application." New York: John Wiley and Sons.
18. Pedhazur EJ (1984): "Multiple Regression in Behavior Research," 2nd ed. New York: Holt, Rinehart and Winston.
19. Ford D, Easton DF, Peto J (1995): Estimates of the gene frequency of BRCA1 and its contribution to breast and ovarian cancer incidence. Am J Hum Genet 57:1457–1462.
20. Horsfall DJ, Jarvis LR, Grimbaldeston MA, Tilley WD, Orell SR (1989): Immunocytochemical assay for estrogen receptor in fine needle aspirates of breast cancer by video image analysis. Br J Cancer 59:129–134.
21. Masood S, Frykberg ER, McLellan GL, Scalapino MC, Mitchum DG, Bullard JB (1990): Prospective evaluation of radiologically directed fine-needle aspiration biopsy of nonpalpable breast lesions. Cancer 66:1480–1487.