Journal of Cellular Biochemistry 25S:112–122 (1996)

Identification of a Chemoprevention Cohort From a Population of Women at High Risk for Breast Cancer

Carol J. Fabian,[1]* Sahar Kamel,[1] Carola Zalles,[2] and Bruce F. Kimler[3]

[1]Division of Clinical Oncology, University of Kansas Medical Center, Kansas City, Kansas
[2]Department of Pathology, University of Kansas Medical Center, Kansas City, Kansas
[3]Department of Radiation Oncology, University of Kansas Medical Center, Kansas City, Kansas

Abstract In a prospective pilot study, we performed breast fine needle aspirations (FNAs) on 213 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 were those with a first degree relative with breast cancer (73%), prior biopsy indicating premalignant breast disease (26%), a history of breast cancer (13%), or some multiple of these risk factors (11%). Median ages of the high-risk and low-risk groups were 44 and 42, respectively. Sixty-three percent of the high-risk and 73% of the low-risk group were premenopausal. Sixty-eight percent of the 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 25%, 36%, and 28% of high-risk subjects but in less than 4% of low-risk subjects ($P < .0002$). Overexpression of ER and HER-2/*neu* occurred in 8% and 19%, respectively of high-risk women; no low-risk women had these abnormalities. Sixty-eight percent of high-risk women and 7% of low-risk women had abnormalities of one or more of these biomarkers exclusive of cytology. Thirty-one percent of high-risk women, but no low-risk women had abnormalities of two or more biomarkers ($P = .0004$). Biomarker abnormalities were more frequent with increasing cytologic abnormality. Eighteen percent of women with normal cytology, 29% of women with epithelial hyperplasia and 60% of women with hyperplasia with atypia had abnormalities of two or more biomarkers ($P = .048$ and $<.0001$, respectively). Restricting the analysis to those three biomarkers most frequently overexpressed in the high-risk group (ploidy, EGFR, p53), 13% of high-risk women with normal cytology, 20% of high-risk women with epithelial hyperplasia and 51% of high-risk women with atypical hyperplasia had abnormalities of 2 or more of these 3 biomarkers. At a median follow up of two years, 8 of 213 women have been diagnosed with in situ (n = 5) or invasive (n = 3) cancer. Later detection of neoplasia was associated with prior FNA evidence of atypical hyperplasia ($P < .0001$) and multiple biomarker abnormalities in the 5 test battery ($P = .006$) by univariate analysis. By multivariate analysis, development and/or detection of cancer was primarily predicted by atypical hyperplasia ($P = .0047$) and secondarily by multiple biomarker abnormalities ($P = 0.021$). Atypical hyperplasia, EGFR, and p53 in breast FNAs have promise as risk markers and as surrogate endpoint biomarkers for breast cancer chemoprevention trials. J. Cell. Biochem. 25S:112–122. © 1997 Wiley-Liss, Inc.

Key words: biomarkers; breast cancer; chemoprevention; high-risk

INTRODUCTION

Morphologic and molecular tissue markers are needed to identify individuals at high short-term risk for breast cancer development. Risk biomarkers which are reversible could be used as surrogate response indicators in short term chemoprevention trials.

Optimal candidates for risk and surrogate response biomarkers are those that reflect fundamental mechanisms involved in epithelial carcinogenesis such as proliferation, dysregulated growth and abnormal DNA repair activity [1]. The ideal biomarker would be one that is: 1) highly sensitive and specific for future breast cancer development, i.e., predicts future breast cancer in 25% or more of instances, 2) readily detected by random sampling of precancerous tissue, 3) amenable to repeated sampling, 4) readily quantitated and easily performed in a variety of laboratories, 5) present in most precancerous tissue regardless of the type of predisposing genetic factors, initiating event, or promotional influences, 6) reversible with successful chemopreventive treatment and 7)

*Correspondence to: Carol J. Fabian, MD, Professor of Medicine, Division of Clinical Oncology, University of Kansas Cancer Center, 3901 Rainbow Boulevard, Kansas City, KS 66160–7820.

Received 22 January 1996; Accepted 30 April 1996

© 1997 Wiley-Liss, Inc.

modulated by a wide variety of chemopreventive agents [2–4].

There is no single ideal risk and surrogate response biomarker for breast cancer. To date, the most specific biomarkers for breast cancer development are germline mutations of certain tumor suppressor genes (e.g., BRCA1, 2 and 3 and p53) for hereditary breast cancer and histologic changes of lobular and ductal carcinoma in situ [5]. Unfortunately, germline genetic changes are present in 5% or less of women who will eventually develop breast cancer and are not reversible [6]. Carcinoma in situ is a late and often focal event. Moreover, repeated breast biopsies to sample breast carcinoma in situ over a protracted period of time pose several logistical problems.

Hyperplasia, with or without atypia, while not as specific for later cancer development, as is in situ carcinoma, is associated with increased risk [5], occurs earlier in the carcinogenic process and may be more diffusely distributed. Thus, hyperplasia may be more amenable to repeated random sampling than in situ carcinoma, especially in populations epidemiologically defined as at increased risk. Marshal et al. using random four quadrant fine needle aspiration, found evidence of moderate to severe hyperplasia in 39% of 51 women with a first degree relative with breast cancer [7]. These investigators postulated that proliferative breast disease may represent an early field change in genetically predisposed women [8].

Multiple genetic as well as epigenetic mechanisms are involved in the tumorigenic process and include oncogene activation and alteration or deletion of tumor suppressor genes or differentiation genes [9–16]. These changes permit a hyperproliferative and dysregulated state [17]. It is unclear how early in breast carcinogenesis these changes take place, but alterations in oncogenes and tumor suppressor gene expression have been found in breast tissue biopsies in which only hyperplasia was noted [9,11–14].

To develop a more accurate means of predicting short-term risk of breast cancer, we initiated a study of breast tissue obtained by random fine needle aspiration. We studied cytologic pattern and molecular markers (ploidy, ER, EGFR, p53, HER-2/*neu*) which are often abnormal or overexpressed in breast cancer. Women were then followed for breast cancer development. Fine needle aspirates were also obtained from a group of very low risk women for comparison.

METHODS

High-risk women were eligible for study if they had any one of three known major epidemiologic risk factors: a) a first degree relative with breast cancer, b) prior precancerous biopsy (atypical hyperplasia or carcinoma in situ) and c) prior breast cancer. Although not used to determine subject eligibility, 10 and 30 year projected probabilities of developing breast cancer were calculated using a modified Gail model [18].

High-risk women were either self-referred or referred by a large number of private physicians. Low-risk controls were paid volunteers recruited through an advertisement in the medical center bulletin. Low-risk controls had no major risk factors for breast cancer, as listed above. In addition, they had no clinical evidence of moderate to severe fibrocystic disease by physical exam or mammography, no second degree relatives with breast cancer, or first or second degree relatives with ovarian cancer. Both high-and low-risk women were required to have a mammogram interpreted as "not suspicious for breast cancer" within 12 months prior to the aspiration. Clinical breast exam performed immediately prior to aspiration also must not be suspicious for breast cancer. Estrogen replacement therapy was permitted. However, for the sake of consistency, women receiving estrogen replacement were required to take progesterone replacement as well for at least one month prior to aspiration. Only the contralateral breast was aspirated in women with prior breast cancer, and at least one year must have elapsed between aspiration and completion of adjuvant chemotherapy or antiestrogen treatment. Women were generally required to be between the ages of 30 and 60. Our rationale for this age range is that the risk of breast cancer development rises sharply after 30 [19], and that breast tissue of women over 60 usually contains a large amount of fat which makes it difficult to acquire an adequate number of epithelial cells. However, exceptions were made for high-risk women younger than 30 if they were within ten years of the age at which their mother or sister were diagnosed with breast cancer. A high-risk woman over 60 could be entered if she had evidence of severe proliferative disease on mammography or if she had a recent precancerous biopsy.

The procedures for aspiration, tissue processing and assay analysis have been detailed else-

where but are summarized in Table I [20,21]. Cytologic criteria for normal, apocrine metaplasia, hyperplasia, atypical hyperplasia or cancer are detailed in Table II [22–24]. One pathologist interpreted all cytology slides (CZ). Periodically, sets of 25 slides were reevaluated without knowledge of prior interpretations. Intraobserver variance was 8% (8/100). In addition, these sets of 25 slides were reviewed by an additional pathologist. Interobserver variance was 14% (10/75). Two reviewers independently interpreted all immunocytochemistry slides. Interobserver variance was 4% [21]. Each woman is initially aspirated twice 6 months apart. Women having FNA evidence of hyperplasia with atypia or hyperplasia with multiple abnormal biomarkers are encouraged to undergo clinical breast exam by a health care professional 3–4 times yearly and mammography twice yearly. Others are encouraged to continue with yearly clinical breast exam and mammography. Women with atypia are reaspirated yearly, others are reaspirated every 2–3 years. All women are contacted yearly by letter or phone.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows (Release 6.1 SPSS Inc., Chicago, IL). *P* values were completed according to Pearson and Hartley [25]. Multiple regression analyses were performed using procedures detailed by Dillon and Goldstein [26]. A stepwise multiple regression equation was constructed for cytologic category and each of the other biomarkers [27]. Modifying variables for cytologic categories included the other biomarkers, menopause, current age, age at first birth, modified Gail risk estimates and risk factor subcategory. When the individual biomarkers were used as the dependent variable, cytologic designation was included as the modifying variable.

TABLE I. Aspiration Methodology

Subjects
- Women age 30–60 during luteal portion menstrual cycle for premenopausal women.

Procedure
- Local anesthetic 4 parts lidocaine; 1 part bicarbonate just medical and lateral to the aerola at 3 and 9 o'clock
- 1½″ 21 gauge needle attached to 12cc syringe prewetted with tissue culture medium. 8–10 aspirates/breast. 4–8 needle passes/aspirate.
- Aspirate material pooled in ice cold tissue culture medium until aliquoted for cytology, ploidy, ER, EGFR, p53, HER-2/*neu*.
- Aspirate kept on ice until fixation.

Methods
- CytologyFiltered, 25 mm millipore 5 μm membrane
- Ploidy Feulgen's stain, image analysis, DNA index ≤0.85 or ≥1.15 considered aneuploid
- ERAbbott-Erica Kit
- EGFRClone F_4 Sigma
- p53PAb240 Oncogene Science
- HER-2/*neu*Ab#3 Oncogene Science

Results
- ≥2+ Staining (10% cells) considered overexpression

Eventual cancer development (i.e., clinical diagnosis of breast cancer previously undetected) as the dependent variable was also analyzed by multiple regression. Modifying variables included were the cytologic category, biomarker results as well as epidemiologic factors listed above. Also performed were regressions using absence or presence 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). The Bonferroni correction was applied to all regression analyses [28].

The present analysis is limited to 213 eligible high-risk and 30 low-risk women who were entered between March 1991 and April of 1995 and who had cytology and all six biomarker assays (including ploidy, ER, EGFR, p53 and HER-2/*neu*) performed or attempted. Comparisons of demographic variables for high- and low-risk subjects are shown in Table III. Both high- and low-risk study groups were predominately premenopausal with a median age of 44 years (range 29–65) in the high-risk group and 42 years (range 31–52) in the low-risk group. Seventy-three percent of women in the high-risk group had one or more first degree relatives with breast cancer. Eighteen percent of the high-risk group had four or more relatives with breast cancer and thus fit the definition of belonging to a hereditary breast cancer family recently proposed by Ford, Easton and Petro [29]. Differences in distribution of demographic factors were notable between the high-risk subgroups. The median age was lowest for women with an affected first degree relative as their only criterion of eligibility, and was highest for those eligible because of a prior node negative breast cancer (42 versus 49). Likewise, while 67% were premenopausal in the family history high-risk subgroup, only 43% were premenopausal in the prior cancer subgroup.

TABLE II. Criteria for Cytologic Morphology Characterization

Type	Overall cellularity and morphology	Cohesiveness	Polarity	Cytoplasm	Nuclei	Cell size
Normal	Scant cellularity Small clusters of oval shaped cells Myoepithelial cells present	Yes	Yes	Abundant	Large, even, dark no nucleoli	Variable
Apocrine metaplasia	Moderate cellularity Monolayer sheets of monomorphic oval, polyhedral or rectangular cells	Yes	Yes	Abundant eosinophilic	Large with nucleoli	10–25 µm
Epithelial hyperplasia	Increased cellularity Monolayer sheets Myoepithelial cells present	Yes	Yes	Moderate homogeneous	Round, vesicular nuclei Distinct frequent nucleoli, chromatin finely granular and uniformly distributed	15 µm
Hyperplasia with atypia (atypical hyperplasia)	Increased cellularity Loosely arranged groups of nonpolarized cells with indistinct borders, few myoepithelial cells	Loss in some cell groups	Loss	Decreased amount increased nuclear/cytoplasma ratio	Conspicuous overlapping nuclei. Chromatin slightly clumped, slight anisonucleosis nucleoli present, may be micronuclei but no macronuclei	Variable
Carcinoma	Increased cellularity Loosely arranged groups of nonpolarized cells with indistinct borders and marked cellular pleomorphism, myoepithelial cells absent	Marked loss	Marked loss	Decreased amount increased nuclear/cytoplasma ratio	Marked anisonucleosis and marked chromatin clumping, macronuclei often present	Variable

RESULTS

Cytology Patterns in the High- and Low-risk Groups

As shown in Table IV, the prevalence of normal nonproliferative cytology was only 32% in high-risk women, as compared to 83% in the low-risk group. Forty-nine percent of high-risk but only 17% of low-risk women had evidence of epithelial hyperplasia, without atypia, in their fine needle aspirates (FNA). Nineteen percent of high-risk and no low-risk women had evi-

TABLE III. Demographic Factor Distribution in Low and High Risk Subgroups

	Low risk (n = 30)	High risk total (n = 213)	High risk* FH (n = 155)	High risk* precan Bx (n = 55)	High risk* prior BrCa (n = 28)	High risk multiple factors (n = 24)
Median age	42	44	42	48	49	48
% Premenopausal	73%	64%	67%	53%	43%	46%
Estrogen replacement	10%	13%	17%	7%	0%	8%
Median 10 year Gail Risk	1%	5%	5%	7%	4%	12%
Median 30 year Gail Risk	5%	15%	15%	18%	13%	30%
No live birth prior to age 30	0%	33%	34%	33%	39%	42%

*FH = family history, Precan Bx = prior precancerous biopsy, Prior BrCa = prior stage I breast cancer.

TABLE IV. Cytology Pattern Distribution in Low and High Risk Women (Total and Subgroups)

Cytologic description	Low risk (n = 30)	High risk total (n = 213)	P value	High risk* FH (n = 155)	High risk* precan Bx (n = 55)	High risk* prior BrCa (n = 28)	High risk multiple factors (n = 24)
Non-proliferative	83%	32%	<.00001	33%	31%	46%	50%
Epithelial hyperplasia	17%	49%	.0009	47%	49%	46%	38%
Atypical hyperplasia	0%	19%	.0096	19%	20%	7%	12%

*FH = family history, Precan Bx = prior precancerous biopsy, Prior BrCa = prior stage I breast cancer.

TABLE V. Distribution of Biomarker Abnormalities in Low and High Risk Subgroups

	Low risk (n = 30)	High risk total (n = 213)	P value	High risk* FH (n = 155)	High risk* precan Bx (n = 55)	High risk* prior BrCa (n = 28)	High risk multiple factors (n = 24)
Aneuploidy	0%	25%	.0033	26%	24%	28%	33%
≥2 + ER	0%	8%	.12	5%	7%	14%	0%
≥2 + EGFR	3%	35%	.0003	36%	44%	31%	46%
≥2 + p53	3%	28%	.0031	27%	36%	28%	37%
≥2 + HER-2/*neu*	0%	19%	.0096	15%	22%	36%	25%
≥1 Abnormal	7%	69%	<.00001	68%	76%	79%	83%
≥2 Abnormal	0%	31%	.0004	29%	38%	36%	46%

*FH = family history, Precan Bx = prior precancerous biopsy; Prior BrCa = prior stage I breast cancer.

dence of hyperplasia with atypia in their FNA. The differences in the prevalence of normal, hyperplastic and dysplastic FNA cytology patterns between high- and low-risk women were all statistically significant ($P < 0.01$).

Although prevalence of atypical hyperplasia was highest in the family history and precancerous subgroups (19% and 20% respectively) and lowest in the prior cancer group (7%), these differences were not statistically significant.

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

Distribution of biomarker expression between the high- and low-risk groups and amongst high-risk subgroups is shown in Table V. The differences in the prevalences of ploidy abnormalities or biomarker overexpression between the high- and low-risk groups were all statistically significant except for ER. ER overexpression was not detected in any of the 30 low-risk women and in only 8% of high-risk women.

Sixty-nine percent of high-risk women but only 7% of low-risk women exhibited one or more biomarker abnormalities. Thirty-one percent of the high-risk women and none of the low-risk women had two or more biomarker abnormalities. There were no significant differences between the high-risk subgroups in the prevalences of single or multiple biomarker abnormalities.

Association of Biomarker Expression with Cytologic Pattern

The prevalence of individual biomarker abnormalities in the high-risk population was significantly associated with the cytologic abnor-

mality (Fig. 1). The association was particularly striking for EGFR and p53. EGFR was overexpressed in 20% of individuals with normal cytology, 38% of those with epithelial hyperplasia and 59% of women with atypical hyperplasia in their fine needle aspirations (P = .015; normal versus epithelial hyperplasia, P = .026; epithelial hyperplasia versus atypical hyperplasia, and P = .00007; normal versus atypical hyperplasia). p53 was overexpressed in 10% of women with normal FNA cytology, 32% of those with epithelial hyperplasia and 51% of those with atypical hyperplasia (P = .0018; normal versus epithelial hyperplasia, P = .047; epithelial hyperplasia versus atypical hyperplasia, and p=.008; normal versus atypical hyperplasia). p53 and EGFR overexpression were strongly predictive of concurrent atypical hyperplasia in the fine needle aspirate (P = .0022).

The prevalence of multiple biomarker abnormalities was also associated with increasing cytologic abnormality (Fig. 2). Sixteen percent of high-risk women with normal cytology, 29% of women with epithelial hyperplasia and 60% of high-risk women with atypical hyperplasia had two or more biomarker abnormalities (P = .049; normal versus epithelial hyperplasia, P = .00008; epithelial hyperplasia versus atypical hyperplasia, and $P < .00001$; normal versus atypical hyperplasia).

Association of Cytologic and Biomarker Abnormalities With Later Cancer Development

At a median follow-up of 24 months for the entire group, eight of the 213 high-risk women have been diagnosed with in situ [5] or invasive [3] breast cancer. Hazard function analysis indicates a projected 12% of women express in situ or invasive breast cancer at five years (Fig. 3).

Table VI details risk category at entry, age, menopausal status, cytologic and biomarker status, and time to cancer detection. The median time to cancer diagnosis was 10.5 months after entry (range 2–48 months). In all instances, diagnostic breast biopsies were prompted by new mammographic abnormalities [5] or a suspicious mass on clinical breast exam [3]. Four of the women developing cancer had one or more first degree relatives as reasons for entry, five had prior LCIS [3] or DCIS [2] and had been treated by excisional biopsy only before study entry. Two of the breast cancers which developed after study entry were lobular carcinoma in situ (LCIS), two had ductal carcinoma in situ (DCIS), one had both LCIS and DCIS, two had invasive lobular cancer, and one invasive ductal cancer. Two of the three invasive cancers were node-positive. Six of the eight women underwent mastectomy or lumpectomy and radiation ± axillary node dissection and systemic

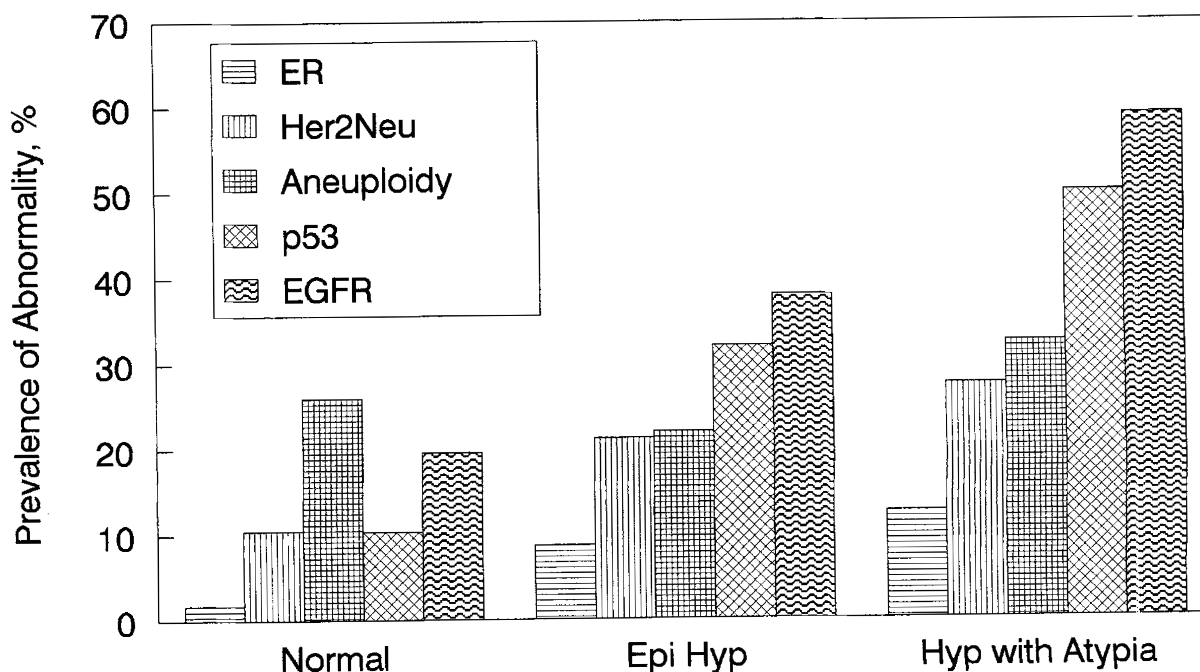

Fig. 1. Prevalence of individual abnormalities in random fine needle aspirates from 213 high-risk women as a function of cytologic category.

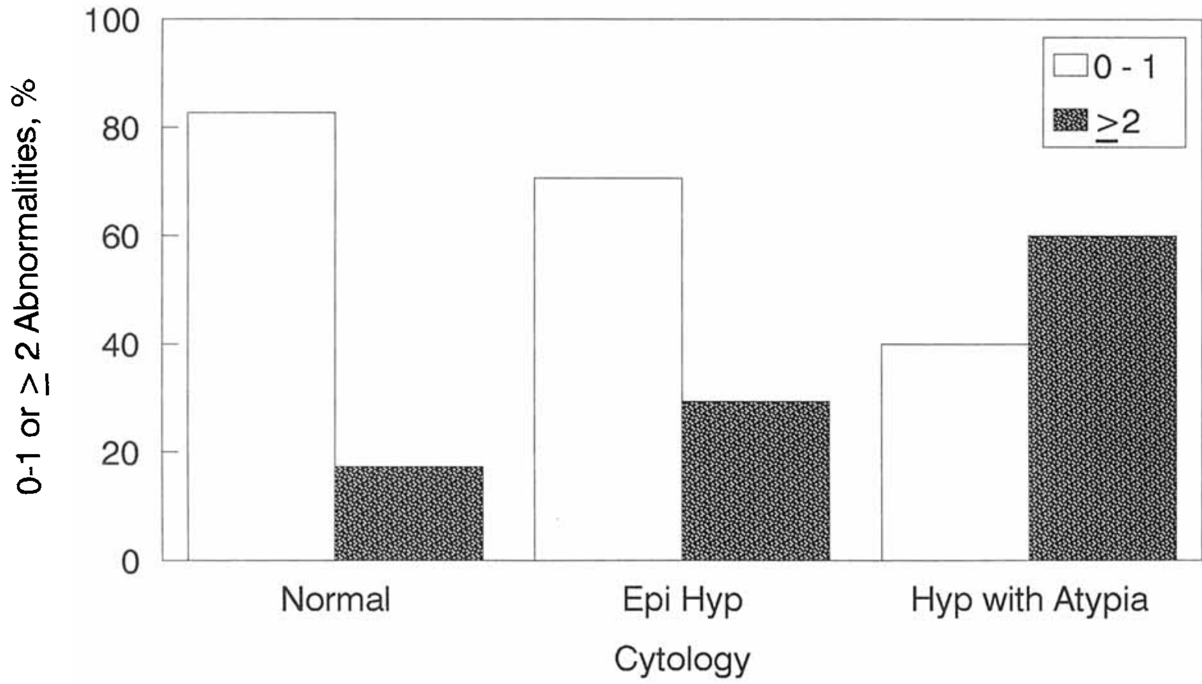

Fig. 2. Prevalence of multiple positive biomarkers in random fine needle aspirates from 213 high-risk women as a function of cytologic category.

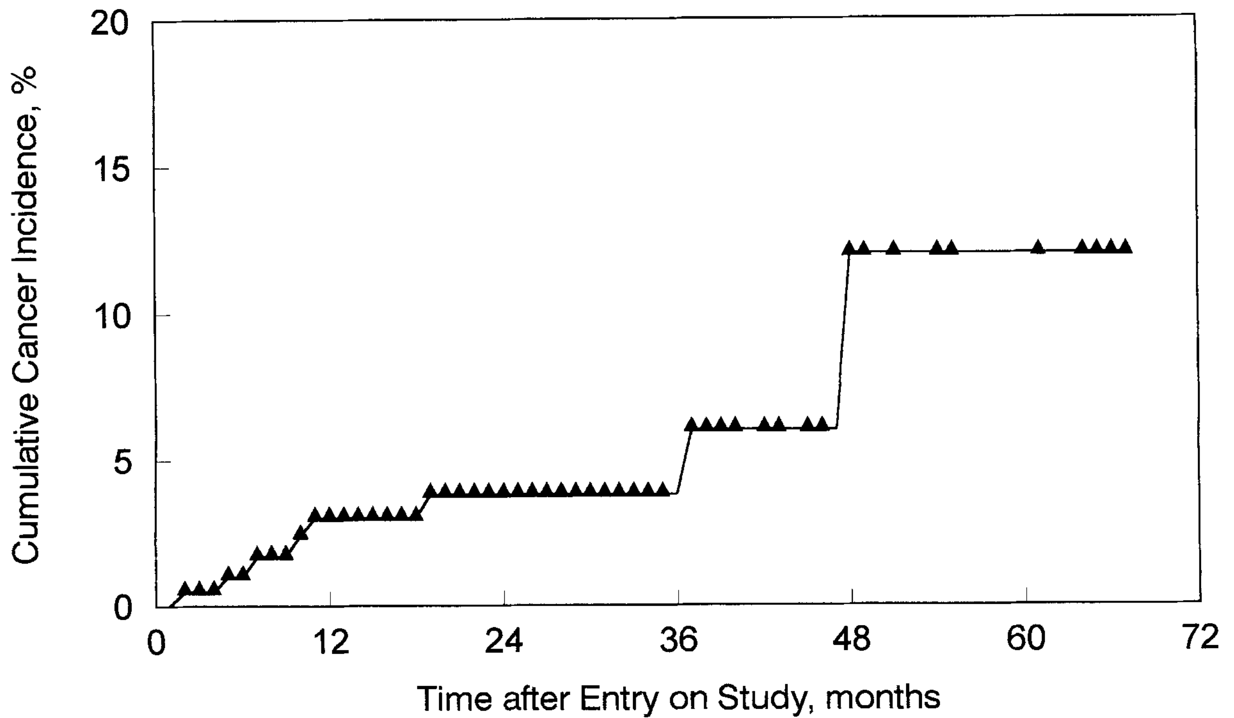

Fig. 3. Hazard analysis of detection of in situ and invasive breast cancer following study entry in 213 high-risk subjects. Median follow-up time of all subjects is 24 months. *Filled triangles:* Times of censoring.

TABLE VI. Characteristics of High Risk Subjects Later Developing Cancer

Age at entry	Meno-pause status	Risk factors	Cytology initial aspirate	Initial abnormal biomarkers	Time to cancer detection (mos)	Type	Stage	How discovered	RX
42	Pre	DCIS + FH	Atypical hyperplasia	EGFR, p53	2	Ductal	T_{is}	MAM	Lumpectomy + XRT
54	Pre	LCIS + FH	Atypical hyperplasia	EGFR, HER-2	5	Lobular	T_{is}	MAM	Mastectomy
51	Post	FH	Atypical hyperplasia	Ploidy, EGFR, HER-2, p53	7	Lobular	T_1N_1	PE	Mastectomy
51	Pre	LCIS	Atypical hyperplasia	None	10	Lobular	T_{is}	MAM	Lumpectomy + follow
53	Post	FH	Atypical hyperplasia	EGFR, p53	11	Lobular	T_3N_1	PE	Mastectomy
65	Post	FH	Normal	p53	19	Ductal	T_1N_0	MAM	Mastectomy
43	Pre	DCIS	Atypical hyperplasia	Ploidy, ER, EGFR, p53	37	Ductal	T_{is}	MAM	Mastectomy
48	Pre	LCIS	Epithelial hyperplasia	Ploidy, ER, EGFR	48	Lobular	T_{is}	PE	Lumpectomy + follow

treatment. The two women with recurrent LCIS elected excisional biopsy and continued follow-up.

Six of the eight high-risk women who later developed cancer had atypical hyperplasia in their initial aspirate. Six subjects had multiple biomarker abnormalities, and seven had either atypical hyperplasia or multiple marker abnormalities. All subjects developing in situ or invasive cancer after their initial aspiration had at least one biomarker abnormality in their initial aspirate. Five of the 24 women with both atypical hyperplasia and two or more biomarker abnormalities have developed breast cancer. One of the 16 women with atypical hyperplasia alone, and one of the 30 women with epithelial hyperplasia and two or more biomarker abnormalities have developed in situ or invasive breast cancer.

By univariate analysis, both atypical hyperplasia and multiple abnormal markers were strongly associated with subsequent cancer development ($P = .00003$ and $.0062$, respectively). In a multiple regression analysis with cancer as the dependent variable and cytology, biomarkers, and Gail risk as independent variables, cancer was again predicted by FNA atypical hyperplasia ($P = .0004$) and multiple positive markers, but the association explained less of the variance ($P = .021$). Neither 10- nor 30-year Gail risk added to the prediction of subsequent breast cancer detection in the multiple regression analysis.

Concurrent FNA atypical hyperplasia in high-risk women was predicted by EGFR overexpression ($P = .0047$), p53 overexpression ($P = .0022$), and multiple abnormal markers whether in the five marker set ($P < .0001$) or when considered as a three marker set of p53, EGFR and aneuploidy ($P = .0001$). Atypical hyperplasia was also associated with premenopausal status ($P = .0017$).

DISCUSSION

This study provides preliminary evidence that breast FNA can be utilized to select, from a group of women considered at high lifetime risk based on epidemiologic factors, a cohort at very high short-term risk for development of in situ or invasive breast cancer. Specifically, cytologic atypical hyperplasia, as well as multiple abnormalities of the molecular markers ploidy, EGFR, p53, ER and HER-2/*neu* were predictive of later breast cancer detection in a high-risk population. Aneuploidy, and overexpressed EGFR and p53 were the most frequently noted abnormal biomarkers in the high-risk population, and EGFR and p53 were the biomarkers most closely associated with concomitant atypical hyperplasia. Thus, p53, EGFR, and ploidy, along with cytology pattern are the most likely candidates for risk and surrogate response biomarkers in chemoprevention trials. This is an interim analysis of an ongoing study and to date the rate of cancer detection exceeds that which we had expected (0.5% per year). This may be due

to the inclusion of a large number of women who probably belong to a hereditary breast cancer family and those who have had prior in situ cancer. Longer follow-up and entry of up to a total of 700–900 subjects will be needed to determine the pattern of biomarker abnormality, which along with cytology, is predictive for cancer development particularly in the high-risk population without prior carcinoma in situ. It is acknowledged that five of eight women did not develop invasive, but rather in situ, carcinoma which is technically precancerous breast disease. If the breast tissue is removed, then there is little chance to develop an invasive cancer. It is possible that a more functional definition of "cancer" for our purposes is either an in situ or invasive event which is thought to require mastectomy or lumpectomy and radiation. All women except two who have to date developed in situ only disease have had bilateral mastectomies with reconstruction. If the data are censored so as to exclude the two women developing recurrent LCIS but who continue to be followed without mastectomy, FNA atypical hyperplasia continues to be a significant predictor of later cancer detection ($P = .006$).

To facilitate validation of cytologic and biomarker parameters, improvements in quantitation and automation are desirable. A semiquantitative cytology grading system such as that proposed by Masood [30] and a weighted scoring system for immunocytochemistry analysis such as that utilized by Grizzle [31] may decrease intraobserver variability. However, true quantitation requires image analysis of nuclear morphology and immunocytochemical staining. Full automation is difficult as FNAs contain a heterogeneous mixture of ductal, myoepithelial, and inflammatory cells. Furthermore, many image analysis systems (we use CAS II) require a monolayer of cells and ductal cells usually form three dimensional clumps. We have found that treatment of breast aspirates with collagenase at a concentration of 250 μg/ml for 10 min allows monolayer formation but may decrease the number of aneuploid specimens. Further, although quantitative measurements of nuclear area, size, shape, optical density and chromatin pattern, as measured by image analysis, tend to be useful in differentiating benign from malignant lesions, they have yet to be validated as risk and surrogate response biomarkers in prospective clinical trials [32–38].

Phase II chemoprevention agents will be compared, in a randomized double blind fashion, to placebo. In intermediate range studies, tissue will be sampled before and after 3–6 months of drug administration. It is important that adequate material be obtained to perform all anticipated tests before and after the study drug. If the drug is effective, proliferation may be decreased, resulting in fewer available cells. In the first two years of the six marker study, cells from the right and left breasts were analyzed separately and results were pooled. Multiple aliquoting resulted in substantial cell loss and inability to satisfactorily perform all six assays from the same aspiration. Consequently, cytology and half of the biomarkers were performed 6 months apart. The combined results of these two aspiration settings constituted the initial aspiration.

In the past year, we have simplified our methodology so that cells from both breasts are pooled from both breasts and all six assays are performed from one instead of two aspiration settings. Thus, aspirations can be repeated at 6 month intervals such as would be needed for a phase II chemoprevention trial. In 43 patients assessed by this protocol, the QNS (quantity not sufficient) rate for any of five biomarker tests after aspirate was 30% for patients with normal cytology ($n = 13$), 16% for patients with epithelial hyperplasia ($n = 19$), and 6% for those with atypical hyperplasia ($n = 11$). To reduce the QNS rate even further, we recommend eliminating at least one or two biomarker (i.e., ER and/or HER-2/*neu*) tests from the panel if FNA is to be used to monitor breast tissue changes in Phase II chemoprevention trials.

In summary, later detection of neoplasia was associated with prior evidence of atypical hyperplasia in breast FNAs. EGFR and p53 are predictive of concurrent atypical hyperplasia. Atypical hyperplasia, EGFR, and p53 in random breast FNAs have particular promise as risk and surrogate response markers in chemoprevention trials.

REFERENCES

1. Mulshine JL, Jett M, Cuttitta F, Treston AM, Quinn K, Scott F, Iwai N, Avis I, Linnoila RI, Shaw GL (1993): Scientific basis for cancer prevention. Intermediate cancer markers. Cancer 72 (Suppl):978–983.

2. Kelloff GJ, Boone CW, Steele VE, Fay JR, Lubet RA, Crowell JA, Sigman CC (1994): Mechanistic considerations in chemopreventive drug development. J Cell Biochem 20 (Suppl):1–24.
3. Goodman GE (1992): The clinical evaluation of cancer chemoprevention agents: Defining and contrasting phase I, II, and III objectives. Cancer Res 52 (Suppl): 2752s–2757s.
4. Freedman LS, Schatzkin A, Shiffman MH (1992): Statistical validation of intermediate markers of precancer for use as endpoints in chemoprevention trials. J Cell Biochem 16G (Suppl):27–32.
5. Dupont WD, Page DL (1985): Risk factors for breast cancer in women with proliferative breast disease. N Engl J Med 312:145–151.
6. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W (1994): A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 226:66–71.
7. 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.
8. Skolnick MN, Cannon-Albright LA (1992): Genetic predisposition to breast cancer. Cancer 70:1747–754.
9. Millikan R, Hulka B, Thor A, Zhang Y-C, Edgerton S, Zhang X-X, Pei H, He M, Wold L, Melton LJ, Ballard D, Conway K, Liu ET (1995): p53 mutations in benign breast tissue. J Clin Oncol 13:2293–2300.
10. Baylin SB, Herman JG, Wales MM, Vertino P, Wu J, Kurbitz S, Issa JP (1995): Hypermethylation of CpG islands and inactivation of tumor suppressor genes. Proc Am Assoc Cancer Res 36:691.
11. Whittaker JL, Walker RA, Varley JM (1986): Differential expression of cellular oncogenes in benign and malignant human breast tissue. Int J Cancer 38:651–655.
12. Athanassiadou PP, Veneti SZ, Kyrkou KA, Intzes KS, Mouzaka LI, Papadimitriou GK (1992): Presence of epidermal growth factor receptor in breast smears of cyst fluids: Relationship to electrolyte ratios and pH concentration. Cancer Detect Prev 16:113–118.
13. Tsutsumi Y, Naber SP, DeLellis RA, Wolfe HJ, Marks PJ, Mckenzie SJ, Yin S (1990): *neu* Oncogene protein and epidermal growth factor receptor are independently expressed in benign and malignant breast tissues. Hum Pathol 21:750–758.
14. Khan SA, Rogers MAM, Obando JA, Tamsen A (1994): Estrogen receptor expression of benign breast epithelium and its association with breast cancer. Cancer Res 57:993–997.
15. Martino S, Ensley JF, Weaver D, Benitez P, An T, Maciorowski Z, De Braud F, Hassan M, Kukuruga M, Signhakowinta A, Valeriote F (1989): Cellular DNA content characteristics of needle aspirates from patients at high-risk for developing breast cancer. Proc Am Assoc Cancer Res 30:256.
16. Visscher DW, Mieale MA, Crissman JD (1993): Pathological and biological relevance of cytophotometric DNA content to breast carcinoma genetic progression. J Cell Biochem 17G (Suppl):114–122.
17. Boone CW, Kelloff GJ (1993): Intraepithelial neoplasia, surrogate endpoint biomarkers and cancer chemoprevention. J Cell Biochem 17F (Suppl):37–48.
18. 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.
19. Pike MC, Spicer DVV (1993): The chemoprevention of breast cancer by reducing sex steroid exposure: Perspectives from epidemiology. J Cell Biochem 17G (Suppl):26–36.
20. Fabian CJ, Zalles C, Kamel S, Kimler BF, McKittrick R, Trainin AS, Zeiger S, Moore WP, Hassanein RS, Simon C, Johnson N, Vergara G, Jewell WR, Lin F, Bhatia P, Chin T (1994): Prevalence of aneuploidy, overexpressed ER, and overexpressed EGFR in random breast aspirates of women at high- and low risk for breast cancer. Breast Cancer Res Treat 30:263–274.
21. Zalles C, Kimler BF, Kamel S, McKittrick R, Fabian CJ (1995): Cytologic patters in random aspirates from women at high- and low risk for breast cancer. Breast J 1:343–349.
22. Oertel YC (1987): Atypical ductal hyperplasia. In Oertel YC (ed): "Fine Needle Aspiration of the Breast." Stoneham, MA: Butterworths, pp 94–103.
23. Kline TS, Kline IK (1989): High risk lesion. In Kline TS (ed): "Guides to Clinical Aspiration Biopsy." New York: Igaku-Shoin, pp 235–248.
24. Masood S, Frykberg ER, McLellan GL, Scalapino MC, Mitchum DG, Bullard JB (1990): Prospective evaluation of radiologically directed fine-needle aspiration biopsy of nonpalable breast lesions. Cancer 66:1480–1487.
25. Pearson ES, Hartley HO (eds) (1970): "Biometrika Tables for Statisticians," 3rd ed., Vol. 1. Cambridge, MA: Cambridge University Press.
26. Dillon WR, Goldstein M (1984): "Multivariate Analysis Methods and Application." New York: John Wiley and Sons.
27. Pedhazur EJ (1984): "Multiple Regression in Behavior Research," 2nd ed. New York: Holt, Rinehart and Winston.
28. Meyers JL (1979): "Fundamentals of Experimental Design," 3rd ed. Boston, MA: Allyn and Bacon, pp 298–300
29. 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.
30. Masood S (1995): Cytomorphology of fibrocystic change, high-risk, and premalignant breast lesions. Breast J 1:210–221.
31. Grizzle WE, Myers RB, Arnold MM, Srivastava S (1994): Evaluation of biomarkers in breast and prostate cancer. J Cell Biochem 19:259–266.
32. King EB, Chew KL, Hom JD, Duarte LA, Mayall B, Miller TR, Neuhaus JM, Wrensch MR Petrakis NL (1991): Characterization by image cytometry of duct epithelial proliferative disease of the breast. Mod Pathol 4:291–296.
33. King EB, Chew KL, Duarte L, Hom JD, Mayall BH, Miller TR, Petrakis NL (1988): Image cytometric classification of premalignant breast disease in fine needle aspirates. Cancer 62:114–124.

34. Van Diest PJ, Baak JPA (1993): Diagnostic and prognostic implications of morphometry and DNA cytometry in breast cytology. In Mouriquand J (ed): "Diagnosis of Non-palpable Breast Lesions: Ultrasonographically Controlled Fine Needle Aspiration." New York: Karger, pp 43–53.
35. Mouriquand J, Bouchet Y, Sage JC, Mermet MA, Ecochard R, Parsi B, Van Diest PJ (1993): Cytoprognostic classification of breast carcinomas: Its predictive value. In Mouriquand J (ed): "Diagnosis of Non-palpable Breast Lesions: Ultrasonographically Controlled Fine Needle Aspiration." New York: Karger, pp 37–42.
36. Bacus JW, Bacus JV (1994): Quality control in image cytometry: DNA ploidy. J Cellular Biochem 19 (Suppl): 153–164.
37. Bacus JW, Bacus JV (1994): A method of correcting DNA ploidy measurements in tissue sections. Mod Pathol 7:652–664.
38. Dawson AE, Cibas ES, Bacus JW, Weinberg DS (1993): Chromatin texture measurement by markovian analysis. Use of nuclear models to define and select texture features. Anal Quant Cytol Histol 15:227–235.