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Biomarker and Cytologic Abnormalities in Women at High and Low Risk for Breast Cancer

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Abstract Fine needle aspirates (FNA) from 106 high-risk women and 25 low-risk women were evaluated for overexpression of estrogen receptor (ER), epidermal growth factor receptor (EGFR), mutant p53, and HER-2/*neu* by immunocytochemistry, and for aneuploidy by image analysis. Aspirates were also classified cytologically as normal, apocrine metaplasia, epithelial hyperplasia (EH), or dysplasia. High-risk women were those with a first-degree relative with breast cancer (76%), precancerous breast disease (26%), prior cancer of the contralateral breast (9%), or multiple abnormalities (11%). Low-risk women had none of the above risk factors, nor a prior breast biopsy or clinical evidence of fibrocystic disease. The median 10-year Gail risk for the high-risk group was 4%, compared to 0.7% for the low-risk group. There were significant differences ($p < 0.01$) between high- and low-risk women in the prevalences of hyperplasia (55% versus 12%), dysplasia (19% versus 0%), aneuploidy (32% versus 0%), overexpressed EGFR (32% versus 4%), and overexpressed p53 (29% versus 4%). The prevalence of multiple biomarker abnormalities was also greater in high-risk than in low-risk women (28% versus 0%; $p < 0.01$). Four percent (4%) of FNAs from high-risk women with normal cytology, 29% of aspirates with hyperplastic cytology, and 60% of those with dysplasia were associated with two or more biomarker abnormalities. The differences in the prevalence of multiple biomarker abnormalities among various cytologic categories were statistically significant ($p = 0.02$, normal versus EH; $p = 0.02$, EH versus dysplasia; $p < 0.01$, normal versus dysplasia). Further study of these tissue biomarkers as potential intermediate-term (5–10 year) predictors of breast cancer development is warranted.

Key words: Aneuploidy, biomarkers, breast cancer, dysplasia, epidermal growth factor receptor, estrogen receptor, fine needle aspirates, HER-2, high-risk, hyperplasia, p53

We chose to study cytology, ploidy, and overexpression of estrogen receptor (ER), epidermal growth factor receptor (EGFR), p53, and HER-2/*neu* as potential tissue-derived predictors of later breast cancer development. Our rationale in selecting these for study is that abnormalities of all these variables are present in ≥40% of cases of

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in situ or invasive breast cancer, but in only <10% of normal mammary tissue [1–8]. Moreover, epithelial hyperplasia and atypical hyperplasia are known to be associated with an increased risk of breast cancer [10]. The prevalence of abnormal biomarker expression in proliferative breast disease and/or precancerous lesions is, at present, incompletely defined. We thought it was logical to test multiple abnormalities, as breast cancer is heterogeneous with respect to biomarker expression, and precancerous ductal tissue might be heterogeneous as well [11].

The purpose of this pilot study was to determine the prevalence of biomarker abnormalities in high-risk as compared to very low-risk women, and whether the prevalence of one or more of these biomarker abnormalities correlated with increasing cytologic abnormality. The long-term objective of the study was to identify, at a premalignant stage, those women in a high-risk population who will develop breast cancer between 5 and 10 years from initial testing.

METHODS

Ductal cells were obtained by fine needle aspiration (FNA) under local anesthesia, as described previously [12]. Approximately 8 aspirations were performed per breast, just medial and lateral to the areola. The needle was placed almost perpendicular to the chest wall and tissue behind the nipple was probed deeply in an attempt to sample the terminal ducts. Premenopausal women were aspirated in the luteal portion of their cycles. Aspirations were performed in two separate settings, 6 months apart, to obtain sufficient cells for all tests.

Eligible high-risk women were those who had a first-degree relative with breast cancer, prior node-negative breast cancer, or precancerous mastopathy (atypical hyperplasia or carcinoma *in situ*). Eligible low-risk women were paid volunteers who had none of the above risk factors, no prior breast biopsies or clinical evidence of fibrocystic disease, and who had their first child before age 30.

All women were required to be between the ages of 30 and 60. Women over the age of 60 were eligible if they had prior evidence of epithelial hyperplasia with or without atypia. Estrogen replacement therapy was permitted only if it was combined with progesterone replacement. Women with prior breast cancer who had previously received tamoxifen or adjuvant chemotherapy must have been off these drugs for at least one year prior to the initial aspiration.

Cytology slides were fixed, Pap-stained, and classified as normal, apocrine metaplasia, epithelial hyperplasia, or dysplasia [12–15]. The term dysplasia was used instead of atypical hyperplasia or proliferative breast disease with atypia because of the controversy surrounding use of the term atypical hyperplasia to classify cytology specimens obtained by FNA [16].

Image analysis (CAS) was used for ploidy determination, as this offered the advantage of analyzing only epithelial cells. Cells were fixed and Feulgen-stained per standard CAS instructions. A CAS calibration slide was used for each run as a control, and 100 ductal cells were assayed for each breast. Conservative DNA indices (DIs) of <0.85 or >1.15 were considered indicative of aneuploidy [17].

ER, EGFR, p53, and HER-2/*neu* were determined immunocytochemically. Cytospins were made on poly-*L*-lysine coated slides. ER was assayed using the ERICA kit (Abbott); control slides were supplied with the kit. EGFR was assayed using a monoclonal antibody (Sigma) and a Vecta-stain alkaline phosphatase kit (Vector Labs). MCF-7 cells (Michigan Cancer Foundation) were used as a positive control for EGFR assays. A non-reacting antibody was used as a negative control. p53 was assayed using a monoclonal antibody to mutant p53 (PAb 240, Oncogene Science) and an indirect immunoperoxidase staining kit (Vector Labs) [18]. BT-474 and MDA-453 cell lines (ATCC) were used as positive and negative controls, respectively [19, H. Smith, personal communication]. HER-2/*neu* was assayed using a monoclonal antibody (c-*neu*, Ab-3, Oncogene Science) and an indirect immunoperoxidase visualization method. SK-BR3 cell line (ATCC) was used as a positive control for HER-2/*neu* [20], with a non-reacting antibody used as a negative control.

Ductal cells were scored visually from 0–3+ for staining intensity. Slides with any ductal cell clumps with >2+ were considered positive [5,21]. Although the definition of immunopositivity varies from one study to another, >10% immunostaining of the total number of cells is generally considered positive [22,23]. When we rescored slides according to the percentage of ductal cells

staining positive, we found that ≥2+ positivity of any clump correlated with >10% positivity of all ductal cells. Immunocytochemical assays were scored independently by two investigators (Kamel and Zeiger). Interobserver variability between negative and positive scoring was 4%.

Samples with only stroma or insufficient ductal cells for rating of a given test were given a "QNS" rating for that particular test. Eighteen percent (18%) of tests in women with normal cytology, 9% of tests in women with epithelial hyperplasia, and 7% of tests in those with dysplasia were rated as QNS. To avoid skewing results, both QNS tests and negative tests were considered negative, *i.e.*, lack of evidence of an abnormal test. All 106 subjects were included in the denominator to determine proportional test positivity, even if they were rated QNS for one or more tests.

Ten- and thirty-year risk estimates (projected probability of developing breast cancer) were obtained by using a modified Gail model, as previously described [12,24]. All risk assessments were based on information available before the first breast aspiration. The calculated Gail risk was not used to determine patient eligibility.

When the study was initiated in 1989, parameters tested were cytology, ploidy, ER, and EGFR. p53 was added in March, 1991 and HER-2/*neu* in December, 1991. We report here only those 106 high-risk women aspirated since 1991, for whom all 6 assays (5 biomarkers plus cytology) were attempted and completed as of July 1, 1993.

RESULTS

Subject Demographics

The high-risk group had a median age of 45, and the most frequent reason for study entry was one or more first-degree relatives with breast cancer. The median age of the low-risk group was 39. Comparison of subject demographics is shown in Table I.

Prevalence of Biomarker Abnormalities: Differences Between High- and Low-Risk Groups

In the high-risk group, the prevalence of aneuploidy was 32%, ER overexpression 10%, EGFR overexpression 32%, p53 overexpression 29%, and HER-2/*neu* overexpression 12% [See Table II for 95% confidence intervals (CI)]. Fifty-nine percent (59%) of the aneuploid cases were hypodiploid, 28% hyperdiploid, and 12% had both hypo- and hyperdiploid peaks. In contrast, there were no abnormalities of ploidy, ER, or HER-2/*neu* in the low-risk group, and only 4% overexpressed EGFR or p53. None of the low-risk women had abnormalities of more than one test. These differences between high- and low-risk

TABLE I. Subject Demographics

	High-Risk (n = 106)	Low-Risk (n = 25)
1st Degree Relative	76%	0
Precancerous Mastopathy	26%	0
Prior Contralateral Breast Cancer	9%	0
Multiple Risk Factors	11%	0
1st Birth After 30 or Nulliparous	33%	0
Estrogen Replacement Therapy	12%	0
Premenopausal	64%	84%
Median Age	45	39
Median 10 Year Gail Risk	5%	0.7%
Median 30 Year Gail Risk	15%	4%

TABLE II. Prevalence of Biomarker Abnormalities in 106 High-Risk and 25 Low-Risk Women

Tissue Variable	High-Risk	(95% CI)	Low-Risk	(95% CI)	p-Value
Aneuploidy	32%	(23–42)	0%	(0–14)	0.0004
≥2+ ER	10%	(5–18)	0%	(0–14)	0.09
≥2+ EGFR	32%	(23–42)	4%	(0–20)	0.0045
≥2+ p53	29%	(21–39)	4%	(0–20)	0.0085
≥2+ HER-2/*neu*	12%	(7–20)	0%	(0–14)	0.07
Multiple (≥2) Abnormalities	28%	(20–38)	0%	(0–14)	0.002

TABLE III. Prevalence of Cytologic Abnormalities in 106 High-Risk and 25 Low-Risk Women

Cytologic Category	High-Risk	(95% CI)	Low-Risk	(95% CI)	p-Value
Normal	22%	(14–31)	88%	(69–97)	<0.001
Apocrine Metaplasia	5%	(2–11)	0%	(0–14)	NS
Epithelial Hyperplasia	55%	(45–64)	12%	(3–31)	<0.001
Dysplasia	19%	(12–28)	0%	(0–14)	<0.006

NS = non-significant

women were statistically significant ($p < 0.01$), except for ER ($p = 0.09$) and HER-2/*neu* ($p = 0.07$).

Cytologic Abnormality in High- and Low-Risk Women

The prevalence of normal (non-proliferative) cytology was only 22% in the high-risk group. Fifty-five percent (55%) of high-risk women had epithelial hyperplasia and 19% had dysplasia (See Table III for 95% CI). In contrast, 88% of low-risk women had normal cytology, 12% had epithelial hyperplasia, and none had dysplasia (Table III). The differences in prevalences of normal cytology, hyperplasia, and dysplasia between high- and low-risk women were all statistically significant ($p<.01$).

The prevalences of all biomarkers were greater in women with hyperplastic and dysplastic cytology than in those with normal cytology. Differences were statistically significant for aneuploidy, overexpressed EGFR, and p53, whether a comparison was made between normal and hyperplasia or normal and dysplasia ($p < 0.01$). The prevalence of overexpressed ER was also significantly higher in dysplastic specimens ($p = 0.03$) than in those with normal cytology (Fig. 1).

Inter-Association of Abnormal Biomarkers in High-Risk Women

HER-2/*neu* overexpression, ER overexpression, and dysplastic cytology occurred as "isolated" abnormalities in only 15–18% of instances. Isolated abnormalities of aneuploidy (41%), overexpressed EGFR (38%), and overexpressed p53 (29%) occurred more frequently. Isolated biomarker abnormalities of all types, however, were generally associated with epithelial hyperplasia. There was a strong correlation of p53 with HER-2/*neu* ($p < 0.01$), and dysplastic cytology with overexpressed EGFR and p53 ($p < 0.01$).

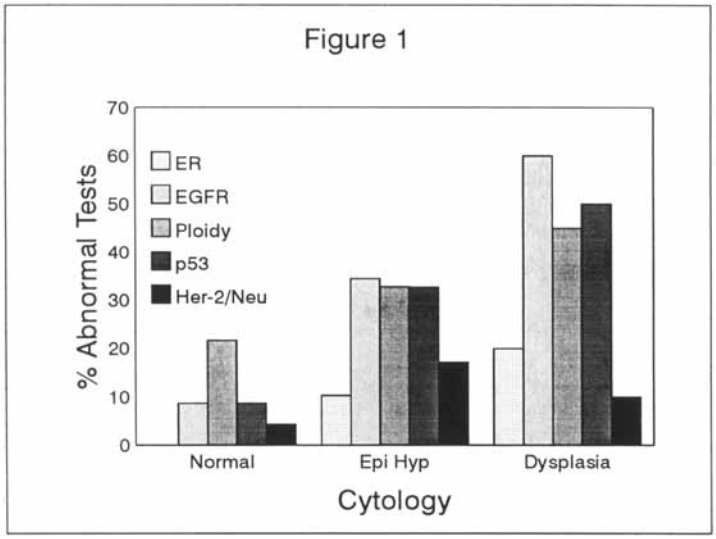

Fig. 1. Correlation of all Biomarkers with Cytologic Category

Prevalence of Multiple Biomarker Abnormalities in High- and Low-Risk Women

Seventy-two percent (72%) of high risk women, as opposed to 8% of their low risk counterparts, had at least one biomarker abnormality exclusive of cytology. Twenty-eight percent (28%) of high-risk women, but none of the low-risk women, had two or more abnormalities. Fourteen percent (14%) of high-risk women had 3 or more, and 4% had 4 or more abnormalities. The differences in prevalences of one, two, or three biomarker abnormalities between high- and low-risk women were statistically significant ($p < 0.01$, <0.01, <0.04, respectively).

Correlation of Multiple Biomarker Abnormalities With Increasing Cytologic Abnormality in High-Risk Women

Multiple biomarker abnormalities appeared to be associated with increasing cytologic abnormality. Only 4% of women with normal cytology had two or more biomarker abnormalities as compared to 29% of women with epithelial hyperplasia and 60% of women with dysplasia. The differences in multiple abnormal biomarker prevalence between normal and hyperplastic ($p = 0.02$), hyperplastic and dysplastic ($p = 0.02$), and normal and dysplastic ($p < 0.0001$) aspirates were all statistically significant by univariate analysis. Multivariate analysis utilizing ≥2 abnormal biomarkers as the dependent variable, and dysplasia, hyperplasia, and the individual biomarkers as modifying variables, showed dysplasia and hyperplasia to be significant predictors of multiple test abnormality.

Correlations of FNA Results With Histologic Sections

One of the 106 high-risk women elected to have bilateral prophylactic mastectomy, and serial frozen sections were obtained from her breast tissue for histologic evaluation and ER, EGFR, p53, and HER-2/*neu* immunohistology. Previous FNA showed dysplasia and overexpression of all biomarkers. Histologic sections showed heterogeneous distribution of normal, hyperplastic, and atypical ductal architecture with a single focus of carcinoma *in situ*. Histologic sections also showed a heterogeneous distribution of overexpression of all the above biomarkers ranging from none, with normal ductal architecture, to strong, with some hyperplastic or dysplastic ducts. Strong HER-2/*neu* staining was seen only in dysplastic ducts.

DISCUSSION

Our results imply that ploidy abnormalities and overexpression of EGFR and p53 may occur fairly early in neoplastic progression. These findings are in agreement with recent work of others who have also reported ploidy abnormalities and overexpression of EGFR and p53 in hyperplastic and/or dysplastic lesions. Martino *et al.* [25] found a 50% prevalence of ploidy abnormalities in FNA of high-risk women. Cohen [26] has postulated that one of the earliest steps in carcinogenesis is loss of tumor suppressor activity through point mutations or deletions. This hypothesis is supported by Visscher's [27] preliminary data in precancerous lesions, which indicate early loss followed by gain of chromosomal material by endoreduplication. Thus, during progression, clones which were originally hypo*diploid may* later become either diploid or hyperdiploid. EGFR overexpression has been previously reported in 12/17 women with epithelial hyperplasia and/or apocrine metaplasia [28].

In our series, the 50% (10/20) prevalence of p53 in dysplastic aspirates is similar to that reported by Bartek *et al.* [29] for invasive breast cancer using frozen tissue and PAb 240 or 1801 monoclonal antibodies. In Bartek's experience, the prevalence of p53 overexpression was greater in frozen than in formalin-fixed, paraffin-embedded breast tumor tissue. At least for PAb 240, there was strong focal staining for mutant p53 in the dysplastic epithelium associated with p53-positive invasive tumors [29]. Thor *et al.* [5], using a high dilution of PAb 1801 in formalin-fixed tissue, found only a 22% p53 immunopositivity in sporadic invasive cancers, and 16% p53 immunopositivity for carcinoma *in situ*. This group of investigators found a 90% specificity for p53 mutations using single stranded conformational polymorphism (SSCP), but only a 77% sensitivity [30]. The prevalence of p53 overexpression as determined by immunohistology is dependent on a number of technical factors, including tissue fixation and type and dilution of antibody used [29–31]. Studies are currently underway to determine specificity and sensitivity of our p53 immunostaining method by performing SSCP analysis and DNA sequencing. Kasten [32] recently postulated that cancers arising from a background of dysplasia may exhibit very early p53 mutations as opposed to those arising from an adenomatous polyp, which may exhibit p53 mutations later in the course of progression.

Finally, it is not clear that FNA cytology accurately reflects the complete histologic picture. In an ongoing prospective trial, breast nipple aspirates showing hyperplasia with atypia have been associated with an increased risk of breast cancer [33]. However, a 15–25% false negative rate has been described in FNA from tumors <2 cm [15, 35].

Despite correlational limitations for FNA cytology, the increased prevalence of single and multiple biomarker abnormalities with increased cytologic abnormalities indicates that one or more of these biological markers may be potentially useful in predicting who will be at highest risk for breast cancer development within a 5–10 year time frame. This hypothesis is markedly strengthened by the highly significant differences observed in the prevalence of aneuploidy, overexpressed EGFR, and p53, as well as hyperplastic and dysplastic cytology between high- and low-risk women.

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