Characteristics of breast ducts in normal risk and high-risk women and their relationship to ductal cytologic atypia.

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Abstract.

Breast ductal cytologic atypia is an important risk factor for sporadic breast cancer**.** Characterization of the associated normal breast tissue is needed to develop additional methods of risk assessment and new targets for breast cancer prevention. We conducted a prospective clinical trial evaluating women at normal risk (NR) or at high-risk (HR) for sporadic breast cancer. Breast ductal cells were collected and studied cytologically and by gene expression profiling, and breast ductal architectural changes were studied by breast ductal endoscopy (BDE) and breast MRI. One-hundred-forty subjects were studied, 70 at HR (RR, 2.0 – 4.6) and 70 at NR. Cytologic atypia was present in 22.9% of HR and 25.7% of NR subjects. Ductal endoscopy was performed in 89 subjects and revealed benign intraductal abnormalities, primarily intraductal fibrous webbing suggesting chronic inflammation, in 40.4% of HR and 5.4% of NR subjects, respectively (P_2 = 0.0002). Two HR subjects with atypia and no NR subjects with atypia developed invasive breast cancer. Gene expression profiling of ductal cells showed comparable gene expression profiles without enriched expression of previously defined oncogenic signatures in subjects with cellular atypia compared to those without atypia, and in HR subjects compared with NR subjects (FDR>0.5**).** Cytologic ductal atypia in normal risk subjects does not appear to be of clinical significance. Atypia in women at high-risk may be associated with benign and malignant breast ductal abnormalities; these characteristics of high-risk ductal cells may not be reflected in gene expression profiles.

Introduction.

Breast cancer is the most common malignancy in women with over 316,000 cases annually in the United States.(1) The risk for sporadic breast cancer may be increased by several factors including hormonal, family history, and histologic changes of breast tissue such as atypical hyperplasia or lobular carcinoma *in situ*. Identification of women at high-risk is important to help with recommendations for the use of surveillance imaging measures, for lifestyle changes, and for consideration of breast cancer prevention methods. Determination of breast cancer risk is commonly made with the aid of risk assessment models such as the Gail Index or the Tyrer-Cuzick Index.(2-4) Women considered to be at high-risk by these models, when treated with the antiestrogen tamoxifen, may reduce the risk of breast cancer by 49%,(3) indicating the importance of risk assessment. Unfortunately, 50%-70% of women who develop breast cancer have no identifiable risk factors,(5,6) suggesting that additional methods are needed to identify and classify women at high-risk for breast cancer.

Most breast cancers develop in the epithelial cells lining the breast milk ducts. Cytologic atypical changes of the ductal epithelial cells has been shown to be an important marker of increased risk for breast cancer. Two prospective studies with long-term follow-up have shown independently that women with cellular atypia detected in breast ductal cytology have an approximately five-fold increased relative risk of developing breast carcinoma.(7-9) The presence of cytologic atypia of ductal epithelium in women at high-risk has been confirmed by many studies, with an incidence of approximately 17% - 35%.(2,7,9-14) This marker is considered to represent carcinogenic progression of the epithelium.(2) One would therefore anticipate that

atypia is accompanied by architectural changes of the breast ducts and genomic charges of the ductal epithelium. If correct, then analysis and characterization of these normal breast tissues may provide important information which could be useful in risk assessment and the development of new prevention targets. Limited studies have been conducted to characterize these tissues with either ductal endoscopy or breast MRI, or with molecular analysis. Two ductal endoscopy studies examined breast ducts in a total of 36 high-risk women with cytologic atypia: they found ductal abnormalities in 19 subjects (52.8%) including 4 cases of atypical hyperplasia, two of radial scar, 11 cases of papilloma and 1 case of DCIS.(10,11) Interestingly, in the study by Cyr et al,(10) intraductal webbing was noted in 3 cases, and on excision one had atypical hyperplasia and two had papillomas, suggesting that the presence of intraductal webbing, while indicating chronic inflammation and proliferation in these ducts, is an important sign of progression in breast carcinogenesis. Three studies evaluated with MRI 38 high-risk women with atypia and found 1 case of ADH and one case of DCIS.(7,11,14) These ductoscopy and MRI studies were performed in high-risk women with atypia, and while indicating intraductal abnormalities of the associated breast epithelium and breast tissue, did not include any women at high-risk without atypia, or any women at normal risk either with or without atypia. These latter studies are needed to better define the relationship of atypia to risk and the presence of associated ductal and breast tissue abnormalities. There are also very limited studies to examine genomic changes in ductal epithelial cells in women with atypia. Fabian et al(9) studied expression of EGFR, ER, p53, and HER-2/Neu in high-risk ductal epithelium and found it was associated with hyperplasia with atypia but did not predict the development of breast cancer. Whole

genome studies such as gene expression profiling are needed to provide important information about underlying epithelial changes associated with atypia and risk.

To address these issues, we have conducted a clinical trial in which women at normal risk or at high-risk for sporadic breast cancer were studied with breast ductal lavage and ductal endoscopy, and identification of women with cytologic atypia further evaluated with breast MRI and gene expression profiling of ductal cells. We now present our findings for the evaluation of 140 women.

Materials and methods

Subject population. All subjects were women participating in an NIH NCI-IRB approved intramural clinical trial (protocol NCI 02-C-0077, NCT 00028340), and all gave written informed consent. The patient studies were conducted in accordance with the ethical guideline U.S. Common Rule. Demographic data were collected for each subject. Risk assessment for breast cancer was conducted with the Gail model and the Tyrer-Cuzick (TC) Risk Assessment model, version 7. Women were defined as being at high-risk if they had a.) a Gail Index $\geq$ 1.67%, or a TC high-risk index $\geq$ 2.0 [the cumulative breast cancer lifetime probability that is $\geq$ double the age- and race-matched general population risk, which was determined from the 2010-2012 SEER database (http://seercancergov/csr/1975-2012/);(4,15)] b.) histologic evidence of lobular carcinoma *in situ* or atypical ductal hyperplasia; or c.) an ipsilateral breast cancer and normal contralateral breast. Women with known deleterious mutations in *BRCA1/2* or other highly-penetrant breast cancer susceptibility genes were eligible for this protocol. All women were required to have a WBC > 2,500, platelet count >50,000, a negative pregnancy test for premenopausal subjects, a negative mammogram within 12 months

for women ≥ 30 years of age, a normal breast examination, and be without any current exogenous estrogen use. Breast cancer subjects must not be currently taking tamoxifen and must have completed chemotherapy at least 4 weeks prior to entry. Women with prior breast irradiation to both breasts, bilateral breast implants, or bilateral major duct excision were not eligible.

Breast ductal lavage (BDL). Breast ductal lavage for the first 59 subjects was performed using a Cytyc microcatheter (Cytyc Corp), and the duct lavaged with 10 ml normal saline as previously described.(2) An aliquot of the final lavage was placed in a ThinPrep vial and analyzed cytologically. The remaining material was separated into cellular and supernatant components with centrifugation at 364 x g, and each frozen at -80^0C.

Breast ductal lavage for subjects 60 – 140 was performed with a modified technique as previously described.(16) This involved placement of a 22G Intracath (Introcan Safety, B Braun Medical, Bethlehem, PA, USA) in the duct, lavage with saline and collection of the lavage materials from the hub of the Intracath. Multiple individual one milliliter samples were collected, a 100 µl aliquot taken from each for cytologic review, and the remaining 900 µl either placed immediately in RLT lysis buffer containing 1% β-mercaptoethanol [BME] (Qiagen Corp, Valencia, CA), vortexed, and frozen at -80^0C, or separated at 364 × *g* at 4°C, and the cellular and supernatant components frozen at -80^0C for future studies.

Breast ductal endoscopy (BDE). Breast ductal endoscopy was performed under intravenous sedation following breast duct lavage, and utilizing an endoscopic unit [Microendoscope (Acueity Corp., Palo Alto, CA)] as described previously.(17) The

endoscopic unit was passed distally until further passage was precluded by narrowing of the duct. Multiple branches were examined; the endoscope was then gradually withdrawn, reexamining the proximal aspects of the duct out to the nipple.

Cytologic analysis and cell count. A 100 μl aliquot of each 1.0 ml ductal lavage sample was taken, placed in PreservCyt, and a ThinPrep slide made for cytopathologic analysis. The cytologic analyses were conducted throughout the duration of the protocol by experienced cytopathologists (mostly ACF), trained and mentored in interpretation of ductal lavage specimens in the early phase of the study. The cytologic diagnostic categories were very similar to the 1997 consensus criteria for breast fine needle aspiration biopsy samples published by the National Cancer Institute, Bethesda, MD.(18) These categories included insufficient cellular material for diagnosis (< 10 ductal epithelial cells), negative for malignancy, atypical (mild atypia or atypia/marked atypia), suspicious for malignancy, or malignant. BDL cases were placed in these categories based primarily on the absence or presence of previously described atypical cytologic features that can be observed in the single ductal dells and/or clusters of ductal cells.(19) The cytologic atypical findings for all subjects were confirmed by one cytopathologist (ACF). In a study by Patil et al,(20) the inter-observer variability for the cytologic diagnosis of BDL samples by pathologists showed good agreement.(20) Epithelial cell counts of each sample were determined as previously described.(17,21)

RNA extraction and amplification. Cell pellets were lysed in 350 μl RLTPlus lysis buffer (Qiagen Corp, Valencia, CA, USA)/BME. Twelve- and one-half micrograms of GenElute™-LPA (Sigma-Aldrich) and 1.5 volumes of 100% ethanol were added to lysates, and the RNA extracted using the RNeasy Micro Kit (Qiagen Corp) according to

the manufacturer's instructions. For the lavage samples in which the initial sample was placed in a 12-mL centrifuge tube as described above, samples were thawed and RNA extracted. Purified RNA was eluted in RNase-free water and analyzed both spectrophotometrically and by an Agilent 2100 Bioanalyzer using an RNA 6000 Pico Chip (Agilent Corp, Santa Clara, CA, USA). RNA preparations were then stored at −80°C.

Gene Expression Microarray: For gene expression profiling, 10 ng of total RNA was amplified and labeled with Ovation Whole Blood Solution kit (Nugen, CA) following the manufacturer's protocol. Labeled cDNA (4.4 ug) of each sample was hybridized to Affymetrix human U133 plus 2.0 GeneChip array at 45 ^{0}C for 16 hrs. GeneChip arrays were washed on Affymetrix Fluidics Station 450 using the manufacturer's recommended scripts and scanned on an Affymetrix GeneChip scanner 3000. Data were collected using Affymetrix AGCC software and normalized with the RNA algorithm.

Bioinformatic analysis. Unsupervised hierarchical clustering was performed on median centered data using uncentered correlation as a similarity metric and centroid linkage. Differential expression of individual genes was evaluated with a Student's t-test. Signature analysis of microarray data was performed with Gene Set Enrichment Analysis (GSEA) software.(22) False discovery rates were calculated using the Benjamini Hochberg procedure.(23) Comparison of clinical and demographic parameters was conducted using Fisher's exact test and Student's t-test analyses.

Results.

Subject characteristics. One hundred forty consecutive subjects were studied from 2002 – 2017. The subject's demographic characteristics are summarized in Table 1.

Among normal risk subjects, 55.1% were at or near a RR of 1.0 by standard risk assessment criteria. Among high-risk subjects, a history of ipsilateral breast cancer was the most common high-risk characteristic; the associated breast cancers were predominantly stage I or stage II. Non-breast cancer subjects were most commonly at high-risk because of elevated Gail Index or Tyrer-Cuzick high-risk index.

Breast ductal lavage and cytologic findings. Breast duct lavage was successfully performed in 139 women; in one subject a duct could not be accessed (Table 2). The cytologic findings are summarized in Table 2. Atypical epithelial cells were identified overall in 34 subjects (24.3%), and were most commonly mild epithelial atypia (17.9%). The overall incidence of epithelial atypia, and the distribution according to mildly atypical cells vs. atypical epithelium was comparable between normal risk subjects and high-risk subjects (*P* = NS; Table 2). The incidence of atypia was also comparable between the two types of catheters used – 25.2% for the Cytyc catheter vs 22.2% for the Intracath catheter. There were no cases of cytology suspicious for, or characteristic of, malignancy in either the normal risk or the high-risk subjects. In the four subjects with a previous diagnosis of ADH the opposite breast was examined and none had atypical cells on ductal lavage cytology.

When the normal risk subjects were stratified according to family history we found, among 18 subjects with atypia, none had a first degree relative with breast cancer, and 3 had a 2nd degree relative (all maternal grandmothers), vs. among 51 subjects with no atypia, where 1 subject had a 1st degree relative (mother) and 1 subject had a 2nd degree relative (aunt). The Incidence of family history was thus minimal and comparable between normal risk subjects with and without atypia. When

stratified according to the relative risk based on TC-risk assessment, among normal risk women with atypia the median TC index was 1.17 (range 0.82 – 1.83); among normal risk women without atypia, the median TC index was 1.21 (range 0.64 – 1.73). The risk assessment index was thus comparable between normal risk subjects with and without atypia. These findings are also comparable to published studies of atypia in lower risk women. In a study by Fabian et al,(9) women at lower risk with a 10-year Gail risk below the median of 4% had no cancers detected in the initial 3 years of follow-up, regardless of their cytology test results. In a study by Wrensch et al,(8) in women without a family history of breast cancer the incidence of atypia was 0.21% with a relative risk (RR) of 3.0, compared to a RR of 1.7 – 2.3 for women without atypia, with overlapping confidence intervals. Among women age 55 years or greater no significant differences were found in the risk of breast cancer by the differing cytologic diagnoses.

Our high-risk study population included 46 women with a prior breast cancer, 16 of whom were previously treated with systemic chemotherapy with or without tamoxifen. To clarify the relationship of atypia to these characteristics, we analyzed the demographic details of the breast cancer patients who had prior systemic therapy, and the 30 women with prior breast cancer who did not have prior systemic therapy; these findings are summarized in Tables 3 and 4, and include the corresponding cytologic findings for each subject. To facilitate comparisons among all subjects in the study, the findings for atypia of all risk groups, both high-risk and normal risk are summarized and compared in Table 5. It can be seen that the incidence of atypia among the high-risk groups is comparable whether or not they had prior systemic therapy. This indicates that the prior systemic therapy was not associated with a higher incidence of atypia. The

findings in Table 5 also indicate that, when the incidence of atypia is analyzed in subgroups of high-risk subjects, the incidence remains comparable to that of normal risk subjects.

Breast ductal endoscopy (BDE). BDE was performed in 89 subjects to define the ductal architectural changes associated with risk and atypia. For the first 84 subjects BDE was performed following BDL. Thereafter, because of the absence of abnormal endoscopic findings in the majority of subjects, BDE was offered for further evaluation only to subjects with atypical epithelial changes on cytologic review at the initial BDL; this included 5 additional subjects. Overall, structural abnormalities of the ducts were noted in 23 subjects (25.8%; Table 6). In all cases these abnormalities were considered to be benign, principally intraductal webbing/fibrous stranding across the ductal lumen. The intraductal webs were present in the major duct as well as in ductal branches. In 3 high-risk subjects yellow friable deposits were noted on the ductal wall which were nonvascular and easily dispersed; two of the three subjects with yellow deposits also had intraductal webbing in the duct. Almost all (91.3%) of the ductal abnormalities were found in the high-risk subjects, with only two normal-risk subjects having intraductal webbing in their ducts (P_2 = 0.0002), and no other abnormalities. A suspicious lesion, either atypical hyperplasia (ADH or ALH) or carcinoma (DCIS or invasive carcinoma) was not seen in any normal risk or high-risk subject. There were no postoperative complications from either the ductal lavage or ductal endoscopy procedures.

The relationship of ductal endoscopic abnormalities to atypia was determined. Twenty-six of 34 subjects with atypia (8 subjects declined endoscopy, all normal risk), and 63 subjects without atypia, were studied (Table 6). Among the normal risk subjects

with or without atypia, only one case of intraductal webbing was noted in each group for an incidence of 10.0% and 3.7%, respectively. Among the high-risk subjects, ductal endoscopy revealed intraductal webs in 37.5% of subjects with atypia and in 33.3% of subjects without atypia (Table 6).

We then examined the relationship of intraductal abnormalities among high-risk subjects according to the presence or absence of a prior breast cancer and prior systemic therapy in a manner analogous to that for atypia (see above and Tables 3,4,5). This revealed that the ductal endoscopic abnormalities were more common in the high-risk groups than in normal risk women whether you included all prior breast cancer women with systemic therapy (P_2 = 0.0002), or excluded subjects receiving chemotherapy within the last 5 years and excluded tamoxifen (P_2 = 0.0083), or excluded all (16) breast cancer subjects with any prior systemic therapy (P_2 = 0.0467; Table 5). The ductal abnormalities in high-risk subjects (principally intraductal webbing) may thus occur independently of prior systemic therapy. It was also noted that, among the high risk subjects, almost all (20/21 – 95.2%) of the intraductal abnormalities occurred in women with a prior breast cancer in the opposite breast (Table 5).

MRI was recommended for further evaluation in all subjects with atypia and was performed in 27 subjects (7 subjects did not have MRI, 5 for technical reasons [implanted metallic devices, body size], and 2 declined). MRI demonstrated abnormalities in 5 subjects, two of which were in high-risk subjects – one case of invasive breast carcinoma and one case of ductal hyperplasia. Three cases of benign abnormalities were found in normal-risk subjects with atypia (fat necrosis, fibrocystic disease, fibroadenoma).

Gene expression profiling of ductal cells.

We conducted microarray gene expression analysis on 29 ductal cellular samples,16 normal risk (9 without atypia and 7 with atypia) and 13 high-risk (8 without atypia and 5 with atypia) to further define the molecular profile associated with risk and atypia. Samples within the respective risk groups for atypia/no atypia were matched according to age and risk. All subjects in the gene expression groups were premenopausal. We performed unsupervised hierarchical clustering of normalized data of all samples. We did not identify any separation of the sample groups (normal risk vs high-risk, or atypia vs nonatypia) according to expression of subgroups of genes. This is illustrated in the heat map of gene expression (Supplementary Fig S1). We then conducted gene expression enrichment analysis of our samples using 3 relevant gene signature sets from the MSigDB Collections, Gene Set Enrichment Analysis (GSEA)**:** Cp [canonical pathways, 1329 gene sets], CGN [cancer gene neighborhoods, 427 gene sets], and C6 [oncogenic signatures, 189 gene sets]. Together this supervised analysis represented a broad array of genes and gene sets involved in cellular metabolism and cancer-related processes. We looked for differences on a gene-by-gene basis in the normal-risk *vs* high-risk groups and in the atypia *vs*. no atypia groups. We found no gene signatures differentially expressed or enriched for either the normal-risk *vs.* high-risk subjects or subjects with atypia *vs*. those without atypia beyond what we would expect by chance alone (false discovery rate > 0.5)

Roman-Perez et al(24) and Troester et al(25), in recent reports, identified two gene expression subgroups in normal breast tissue at high-risk (breast tissue adjacent to cancer) - an Active subgroup associated with overall survival, and an Inactive

subgroup. We obtained the set of genes described in the Roman-Perez et al(24) report that distinguished Active from Inactive microenvironment and applied these to our samples. We conducted unsupervised hierarchical clustering of normalized results using the 3518 genes of the Active/Inactive gene list [Supplementary Table S1,(24)] which we filtered from the composite Affymetrix microarray gene list. The heat map of this clustering is illustrated in Supplementary Fig S2. To further clarify the expression of Active and Inactive gene sets in our samples, we aligned the heat map with a separate column of fold change between the Active and Inactive groups as observed by Roman-Perez et al,(24) with red indicating genes higher in the active tumors and green indicating genes higher in the inactive tumors. We did not see any clear separation of the high-risk vs normal risk groups, or atypia vs. nonatypia groups in our samples according to the Active/Inactive gene sets. We then conducted signature enrichment analysis of our samples using the Active/Inactive gene sets. We did not find any evidence of gene enrichment for the Active sample in either the high-risk or the atypia samples. We did observe enrichment of genes of the Inactive gene set among those with higher expression in the high risk group than the low risk group (P = 0.0322; Supplementary Fig S3, K-S plot), but not in relation to atypia.

Follow-up of subjects with atypia.

On follow-up with a median of 4 years, 4 months (range, 2 months – 15 years/5 months) among all subjects with atypia, one high-risk subject with atypia, a 69-year-old woman with a previous ipsilateral breast cancer, developed an infiltrating carcinoma in the contralateral breast (*i.e.,* the breast that was studied) at 13 years, 3 months. There were no normal risk subjects with atypia who developed breast cancer on follow-up, and

no subjects with atypia, either normal risk or high-risk, who developed ADH or ALH on follow-up.

DISCUSSION.

The present study examined in a comprehensive manner the cytologic, ductal architectural, and gene expression profiling characteristics of the breast ducts and ductal epithelium in women at normal-risk and at high-risk for sporadic breast cancer, and with or without cytologic ductal atypia. This is the first study to examine these characteristic in normal risk subjects. We found cytologic atypia to be present in women at normal risk and at an incidence comparable to that for women at high risk. Studies have previously shown that ductal atypia in high risk women is associated with an increased risk for breast cancer,(8,9) raising the possibility that atypia in normal risk women may also be of prognostic significance. We found, however, that atypia did not correlate with family history or Tyrer-Cuzick risk among the respective normal risk subjects, was not associated with differentially expressed genes on gene expression profiling, was associated with only minimal benign intraductal abnormalities on endoscopy, and was not associated with suspicious breast abnormalities on MRI or on follow-up. Together, these findings indicate that in women at normal-risk for breast cancer, ductal atypia does not appear to be of clinical significance.

In women at high risk, atypia was present at an incidence in agreement with other published series of high risk women (Supplementary Table S1). Atypia is thus not an uncommon finding in women at high risk, and occurs across a range of risk groups and conditions. The ducts of women at high risk were characterized by the presence of intraductal webbing/fibrous stranding and 2 cases of invasive ductal carcinoma. The

incidence of intraductal webbing in subjects at high-risk with atypia was comparable to high-risk subjects without atypia, which suggests that the factors contributing to this abnormality are not necessarily those responsible for the atypia, and that this abnormality may relate principally to the high-risk status. The presence of benign and suspicious abnormalities by ductoscopy and MRI in women at high-risk with atypia has been observed in other series (Supplementary Tables S2,S3). Among all series the overall incidence of ductal abnormalities was 33.1%. Many of these abnormalities were proliferative in nature (radial scar, hyperplasia, papilloma, intraductal webbing, sclerosing adenosis, ADH, DCIS), suggesting a dynamic component associated with these high-risk breast tissues. A striking finding in our study was the presence of intraductal webbing in the high risk breast, which suggests a past history of chronic inflammation. This condition has well established immunosuppressive properties(26,27) and may influence immunosurveillance by the intraepithelial lymphocytes, an important component of ductal epithelium.(28,29) Chronic inflammation is a well-known cause of cancer,(27) and it is reasonable to propose that in these ducts/ductal epithelium already at high risk for breast cancer, that the presence of chronic inflammation could contribute to the carcinogenesis. There are several factors which might be responsible for this chronic inflammation. Multiple studies have confirmed the presence of a microbiome in breast tissues, including normal breast tissue and breast ductal fluid.(30-32) The microorganisms could contribute to chronic inflammation through activation of macrophages,(33) and activation of CD4+ T cells to form Th1 and Th17 T cells,(34) both with release of proinflammatory cytokines and chemokines. Obesity is an important cause of chronic inflammation,(35) as are somatic mutations and environmental factors,

and cell injury and necrosis with release of DAMPS (damage associated molecular patterns).(34) The intraductal webbing in high risk women was almost entirely in women with a cancer in the opposite breast. It has been reported that chronic inflammation is often present in primary cancers and is associated with proinflammatory cytokines including IL-6 and IL-17.(36) High levels of the cytokines IL-6 and IL-17 are also present in the sera of women with breast cancer,(37,38) thus placing them in the position to potentially promote chronic inflammation in the contralateral breast. While speculative, if this supposition is correct it would suggest an important example of the ability of a primary breast cancer to influence the biology of the opposite breast.

When we studied global gene expression profiling in women with and without atypia, we did not identify any differentially expressed genes between the two groups. These findings are in agreement with Ma et al(39) who found that normal breast cells associated with atypical ductal hyperplasia (and thus associated with both atypia and with high risk) do not contain genes differentially expressed from normal risk breast tissue. A comparison of the global gene expression profiles from women at high risk to those at normal risk in our study also did not reveal any significantly differentially expressed genes. This would indicate that the structural abnormalities which have been observed in the high-risk ducts are not reflected in gene expression profiles. It is of interest that gene expression analysis of the Inactive subtype of normal breast tissue described by Roman-Perez et al(24), when studied in our subjects, did show increased activity in the high risk group. These genes are associated with claudins, adhesions, and differentiation. Claudins are proteins associated with tight junctions and control the flow of molecules in the intercellular space between epithelial cells. The downregulation

of several claudins in cancer has been considered consistent with the disruption of tight junctions during tumorigenesis.(40) The increased expression of these genes in the breast ductal cells might suggest a protective function in our high risk subjects.

It is recognized there may also be several reasons why differentially expressed genes were not identified by gene expression profiling in samples from subjects at high-risk or with atypia: a.) the sample size was small; b.) the differences we were looking for were a small subgroup of genes; c.) the population both within and between groups was heterogeneous according to underlying genomic changes and cellular composition, with ductal cell populations including epithelial cells, immune cells and the microbiome.(28,29); d.) the high-risk and normal-risk groups and the atypia and non-atypia groups may not represent pure subgroups to allow identification of gene signatures which have different expression; this possibility is exemplified by the recent finding, using scRNAseq, of three distinct epithelial cell populations in normal breast tissue;(41) e.) the analysis may involve multiple proportions; f.) widespread gene expression changes of some populations of normal breast cells may not occur until later in the carcinogenic pathway. Several reports have identified differentially expressed genes between normal breast tissue adjacent to cancer (NABT) and reduction mammoplasty tissues.(42-44) NABT is at very high-risk for breast tissue [relative risk [RR] 12.0 – 15.0 fold increased risk, (45)]. Our subjects, on the other hand, had a RR of 2.0 – 4.6 fold, which is consistent with the risk of normal breast tissue of most high-risk (for sporadic breast cancer) subjects. It is thus possible that prominent gene expression profiling changes are not observed until higher risk levels than those of our subjects are reached.

Several limitations to the study are noted: 1.) A single duct was usually sampled in the normal risk and high-risk subjects, however multiple samples were taken of each duct, providing more complete sampling of that duct. Examination of 2 or more ducts, and both breasts, might be considered in the future. 2.) The follow-up was short, and the evaluation with MRI was limited to the subjects with atypia; the incidence of development of breast carcinoma in the subjects without atypia is not known. 3.) We acknowledge that the study was not designed to determine the true incidence of breast cancer in the normal risk or the high-risk subjects, which would require a much larger patient population. 4.) The sample size for gene expression studies and for determination of ductal characteristics on follow-up was small.

Summary and conclusions. Cytologic atypia of the breast ductal epithelium is present in women at normal-risk and at high-risk for sporadic breast cancer. Evidence indicates that atypia in the normal risk ducts is not of clinical significance. Multiple ductal abnormalities, both benign and suspicious, are present in the breast ducts at high-risk with atypia and are frequently proliferative and may include lesions at increased risk, at high-risk, or *in* situ or invasive breast cancer. These changes indicate a dynamic nature to the biology of high-risk ducts with atypia. Gene expression profiles of high-risk ductal cells, including those with atypia, may not reflect these ductal characteristics. Analysis of chronic inflammatory changes in the high-risk duct will be an important subject for future studies.

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Table 1. Demographic data.

Category	N
Subjects	140
Normal risk subjects	70 (50.0)*
High-risk subjects: Risk category	70 (50.0)
Breast cancer	46 (32.9)
Gail/Tyrer-Cuzick	19 (13.6)
ADH	4 (2.9)
BRCA1	1 (0.7)
Menopausal status	
Premenopausal	95
Postmenopausal	45
Age	
Normal risk subjects	70
Median age	41 years
Age Range	22-56 years
High-risk subjects	70
Median age	49 years
Age Range	25-71 years
Ethnicity	
Caucasian	58 (41.4)
African American	38 (27.1)
Hispanic	37 (26.4
Asian	7 (5.0)

* Number in parenthesis – percentage of total subjects

Table 2. Ductal lavage and cytologic characteristics

Category	N
Nipple aspirate fluid (NAF)	140 subjects
Absent	90 (64.3)#
Present	50 (35.7)
Normal risk	70
NAF Absent	49 (70.0)
Present	21 (30.0)
High-risk	70
NAF Absent	41 (58.6)
Present	29 (41.4)
Cytologic characteristics of ductal lavage	140 subjects
Negative	96 (68.6)
Mild epithelial atypia	25 (17.9)
Epithelial atypia	9 (6.4)
ICMD	9 (6.4)
Malignant	0 (0.0)
No ductal lavage*	1 (0.7)
Normal risk subjects with atypia	18 (25.7)
Mild atypical epithelium	15 (21.4)
Atypical epithelium	3 (4.3)
High-risk subjects with atypia	16 (22.9)
Mild atypical epithelium	10 (14.3)
Atypical epithelium	6 (8.6)

Number in parenthesis – percentage of total subjects
*Ductal orifice could not be identified.
ICMD – insufficient cellular material for diagnosis

Table 3. Breast Cancer patients with prior systemic treatment.

Subject	Age	Stage breast cancer	ER/PR	HER2/neu	Tamoxifen	Interval since chemotherapy stopped	Interval since tamoxifen stopped	Cytology	Ductal endoscopy
1	65	Stage II	Positive/Positive	Positive	Yes	6 yrs/4 mos	1 yr/11 mos	Atypia	Negative
2	57	Stage II	Negative/Negative	Unknown	No	9 yrs/5 mos		Atypia	Intraductal web
3	58	Stage II	Positive/Positive	Unknown	Yes	11 yrs/6 mos	6 rs/5 mos	Neg	Intraductal web
4	55	Stage II	Negative/Negative	Negative	No	9 yrs/9 mos		Neg	Intraductal web
5	57	Stage II	Positive/Positive	Positive	Yes	5 yrs/5 mos	6 mos	Neg	Intraductal web
6	56	Stage II	Negative/Negative	Negative	No	7 yrs/7 mos		Neg	Intraductal web
7	48	Stage II	Negative/Negative	Unknown	No	6 yrs/2 mo		Atypia	Yellow deposit
8	64	Stage II	Negative/Positive	Unknown	Yes	12 yrs/1 mos	7 yrs/3 mos	Mild atypia	Intraductal web
9	61	Stage II	Positive/Positive	Negative	Yes	8 yrs/1 mos	3 yrs/3 mos	Neg	Intraductal web
10	56	Stage II	Positive/Positive	Negative	Yes	11 yr/7/mos	6 yr/9 mos	Neg	Intraductal web
11	59	Stage II	Positive/Positive	Unknown	Yes	10 yr/6 mo	5 ys/6 mo	Neg	Yellow deposit
12	49	Stage II	Positive/Negative	Unknown	Yes	5 yrs/3 mos	4 mos	Neg	Intraductal web
13*	48	Stage IV	Positive/Positive	Unknown	Yes	3.5 yrs	3.5 years	Not studied	Not studied
14	46	Stage IV	Positive/Positive	Negative	No	3 mos		Neg	Intraductal web
15	68	Stage II	Negative/Negative	Unknown	No	13 yr/4/mo		Neg	Negative
16	63	Stage II	Negative/Negative	Negative	No	9 yr/5 mos		Neg	Negative

*A duct could not be identified

Table 5. Characteristics of High-risk and Normal Risk Subjects

Table 4. Breast cancer patients without prior systemic therapy.

Subject	Age	Stage breast cancer	ER/PR	HER2/neu	Cytology	Ductal Endoscopy
1	33	Stage II	Positive/Positive	Negative	Negative	Not done
2	67	DCIS	Negative/Positive	N/A	Negative	Intraduct web
3	60	Stage II	Positive/Negative	Negative	Negative	Negative
4	51	DCIS	Negative/Negative	N/A	Negative	Negative
5	68	Stage II	Positive/Positive	Positive	Negative	Negative
6	61	Stage II	Positive/Positive	Negative	Atypia	Negative
7	46	Stage I	Positive/Positive	Negative	Mild atypia	Negative
8	65	Stage II	Negative/Negative	Negative	Negative	Negative
9	49	Stage I	Positive/Positive	Negative	Negative	Negative
10	47	Stage II	Positive/Positive	Negative	Negative	Intraduct web
11	45	Stage II	Negative/Negative	Negative	Negative	Negative
12	38	Stage II	Positive/Positive	Positive	Mild atypia	Intraductal web
13	39	Stage II	Negative/Positive	Negative	Negative	Negative
14	41	DCIS	Positive/Positive	N/A	Atypia	Yellow deposit
15	61	Stage II	Positive/Negative	Negative	Mild atypia	Intraduct web
16	51	Stage IV	Positive/Negative	Negative	Negative	Negative
17	61	DCIS	Positive/Positive	Negative	Negative	Negative
18	33	Stage II	Positive/Positive	Positive	Negative	Negative
19	43	Stage III	Positive/Positive	Positive	Negative	Negative
20	56	DCIS	Positive/Positive	N/A	Negative	Intraduct web
21	57	Stage I	Positive/Positive	Positive	Negative	Negative
22	44	Stage I	Negative/Positive	Negative	Negative	Negative
23	49	Stage II	Negative/Positive	Negative	Negative	Intraductal web
24	66	Stage III	Positive/Positive	Positive	Negative	Negative
25	71	Stage I	Positive/Positive	Negative	Mild atypia	Negative
26	46	Stage II	Positive/Positive	Negative	Mild atypia	Negative
27	50	Stage II	Positive/Positive	Positive	Mild atypia	Intraductal web
28	49	Stage I	Negative/Negative	Negative	Negative	Not done
29	45	Stage I	Positive/Positive	Negative	Negative	Not done
30	49	DCIS	Positive/Positive	N/A	Negative	Not done

DCIS – Ductal carcinoma *in* situ; N/A - HER2/neu not determined

Category	Breast Cancer, Prior Systemic Treatment	Breast Cancer, no prior systemic treatment	High-risk Subjects without breast cancer	Normal Risk Subjects	
Total number	16 Subjects	30 Subjects	24 Subjects	70 Subjects	
Age					
Median	57 years	41 years	46	41 years	
Range	46-68	33 - 71	25 - 63	22 - 56	
Stage					
DCIS	0 Subjects	6 Subjects			
Stage I	0	7			
Stage II	14	14			
Stage III	0	2			
Stage IV	2	1			
Atypia					
Negative	11/4#	22	20	52	
Mild Atypia	1/0# (6.7%)/(0.0%)	6 (20.0%)	3 (12.5%)	15 (21.4%)	
Atypia	3/2#(20.0%)/(33.3%)	2 (6.7%)	1 (4.2%)	3 (4.3%)	
Not done	1	0	0	0	
Ductal abnormalities	15 endoscoped/6§	26 endoscoped	12 endoscoped	37 endoscoped	
Negative	3 /2§ (20.0%/33.3%)	18 (69.2%)	11 (91.7%)	35 (94.6%)	
Intraductal webbing	10/3§ (66.7%/50.0%)	7 (26.9%)	1 (8.3%)	2 (5.4%)	
Yellow deposit	2/1§ (13.3%)/16.7%)	1 (3.9%)	0	0	
Not done	1*	4	12	33	
All patients with breast cancer vs. normal risk patients.+					$P_2 = 0.0002$
Breast cancer without chemotherapy within 5 years and without tamoxifen vs. normal risk patients. +					$P_2 = 0.0083$
Breast cancer without any systemic therapy vs. normal risk patients.+					$P_2 = 0.0467$
High-risk without breast cancer vs. normal risk patients.+					$P_2 = 1.000$

***** One subject not studied – a duct could not be identified. # Incidence of atypia after exclusion of subjects with chemotherapy within 5 years and prior tamoxifen. § Incidence of ductal abnormalities after exclusion of subjects with chemotherapy within 5 years and prior tamoxifen. **+** statistical comparisons of ductal abnormalities.

Table 6. Ductal endoscopy findings

Category	N	
Subjects endoscoped	89 subjects	
Normal risk	37 (41.6)	
High-risk	52 (58.4)	
Endoscopic findings	89	
Normal duct	66 (74.2)	
Intraductal webbing/fibrous stranding	20 (22.5)	
Benign yellow friable deposits	3 (3.4)	
Endoscopic findings according to risk		
Normal risk	37 subjects	
Negative	35 (94.6)	
Intraductal webbing/fibrous stranding	2 (5.4)	
Benign yellow friable deposits	0 (0.0)	
High-risk	52 subjects	
Negative	31 (59.6)	
Intraductal webbing/fibrous stranding	18 (34.6)	P_2 = 0.0002 vs. normal risk endoscopic findings
Benign yellow friable deposits	3 (5.8)	
Endoscopic findings according to atypia		
Normal risk with atypia	10 subjects	
Negative	9 (90.0)	
Intraductal webbing/fibrous stranding	1 (10.0)	
Benign yellow friable deposits	0 (0.0)	
High-risk with atypia	16 subjects	
Negative	8 (50.0)	
Intraductal webbing/fibrous stranding	6 (37.5)	
Benign yellow friable deposits	2 (12.5)	
Endoscopic findings according to no atypia		
Normal risk without atypia	27 subjects	
Negative	26 (96.3)	
Intraductal webbing/fibrous stranding	1 (3.7)	
Benign yellow friable deposits	0 (0.0)	
High-risk without atypia	36 subjects	
Negative	23 (63.9)	
Intraductal webbing/fibrous stranding	12 (33.3)	
Benign yellow friable deposits	1 (2.8)	

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