Surgical Oncology 25 (2016) 449–456

Contents lists available at ScienceDirect

Surgical Oncology

journal homepage: www.elsevier.com/locate/suronc

# Cytologic atypia in the contralateral unaffected breast is related to parity and estrogen-related genes

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ARTICLE INFO

*Article history:*
Received 22 October 2015
Accepted 7 December 2015

*Keywords:*
Breast cancer risk
Contralateral unaffected breast
Random fine needle aspiration
Cytologic atypia
Anisonucleosis
Gene expression

ABSTRACT

*Purpose:* The contralateral unaffected breast (CUB) of women with unilateral breast cancer provides a model for the study of breast tissue-based risk factors. Using random fine needle aspiration (rFNA), we have investigated hormonal and gene expression patterns related to atypia in the CUBs of newly diagnosed breast cancer patients.

*Methods:* 83 women underwent rFNA of the CUB. Cytologic analysis was performed using the Masood Score (MS), atypia was defined as MS > 14. RNA was extracted using 80% of the sample. The expression of 20 hormone related genes was quantified using Taqman Low Density Arrays. Statistical analysis was performed using 2-tailed t tests and linear regression.

*Results:* Cytological atypia was more frequent in multiparous women ($P = 0.0392$), and was not associated with any tumor-related features in the affected breast. Masood Score was higher with shorter interval since last pregnancy ($R = 0.204$, $P = 0.0417$), higher number of births ($R = 0.369$, $P = 0.0006$), and estrogen receptor (ER) negativity of the index cancer ($R = -0.203$, $P = 0.065$). Individual cytologic features were associated with aspects of parity. Specifically, anisonucleosis was correlated with shorter interval since last pregnancy ($R = 0.318$, $P = 0.0201$), higher number of births ($R = 0.382$, $P = 0.0004$), and ER status ($R = -0.314$, $P = 0.0038$). Eight estrogen-regulated genes were increased in atypical samples ($P < 0.005$), including TFF1, AGT, PDZK1, PGR, GREB1, PRLR, CAMK2B, and CCND1.

*Conclusions:* Cytologic atypia, and particularly anisonucleosis, is associated with recent and multiple births and ER negative status of the index tumor. Atypical samples showed increased expression of estrogen-related genes, consistent with the role of estrogen exposure in breast cancer development.



## 1. Introduction

A key barrier to the successful implementation of breast cancer prevention strategies is the accurate assessment of breast cancer risk. Established risk models perform well at the population level [1,2], but the discriminatory ability of these models for individual women is modest [3,4]. Better stratification of high risk women would allow the targeting of prevention strategies to those women who are at the highest risk, and separation of women at risk of estrogen receptor (ER) negative from those at risk for ER positive breast cancer would be a significant added advantage. Currently, apart from genetic mutations, the strongest known risk factors are epithelial atypia [5–8] and high mammographic density [8]; each confer relative risks of 4 or more. Epithelial atypia is included in statistical models of risk estimation, but integration of mammographic density into these models has been only modestly successful [9,10]. Despite its established value as a breast cancer risk

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http://dx.doi.org/10.1016/j.suronc.2015.12.001
0960-7404/

marker, only 20% of women with a diagnosis of breast epithelial atypia develop breast cancer [11], and most breast cancer cases have no prior history of breast biopsy, or if biopsied, no diagnosis of atypia [12,13]. Improvement in the performance of atypia as a risk marker is achievable by the combination of cytomorphologic and molecular data in benign breast biopsies [14,15], and by the extension of these findings to women with clinically normal breasts using minimally invasive approaches, such as random fine needle aspiration (rFNA) [6]. Epithelial atypia in rFNA samples implies a similar risk as that posed by histological atypia [6] and is assessed using cytologic scoring systems such as that described by Masood [16]. Molecular analyses of rFNA samples have been used as surrogate endpoints in early phase prevention studies by our group [17] and by Fabian et al. [18]. However, parameters such as gene expression, structural DNA alterations, and epigenetic changes have not been incorporated as predictors of breast cancer risk.

In the present report, we use rFNA of the clinically normal contralateral breast of unilateral breast cancer patients to evaluate cytologic features and expression of hormone-related genes, seeking candidate markers of breast cancer risk. The contralateral unaffected breast (CUB) is at significantly increased risk for future cancer, with similarity in hormone receptor status of index and subsequent contralateral primary tumors [19–21]. Therefore, the CUB is an excellent model for studying not only the early changes that are the setting for the development of malignancy, but also the risk marker profiles related to hormone receptor (HR) positive and HR negative breast cancer subtypes. To test the hypothesis that the HR status of the index tumor is correlated with the cytologic and gene expression profiles of the CUB, we enrolled women with newly diagnosed unilateral breast cancer who were proceeding to primary surgical treatment.

## 2. Methods

### 2.1. Patients and procedures

Eighty-five women undergoing surgery for unilateral breast cancer (2006–08) consented to participate; rFNA of the CUB was performed in the operating room, at the time of the therapeutic procedure. Patient demographic information, risk factors and primary tumor characteristics were recorded (race, parity, interval since last birth, menopausal status, number of primary and secondary relatives with breast cancer and number of previous breast biopsies). Tumor characteristics included in situ or invasive disease, grade, hormone receptors, and HER2 status.

The rFNA of the CUB was performed using a 22-guage needle at two sites (upper outer and upper inner), 8–10 passes per breast [6]. If contralateral prophylactic mastectomy was planned, rFNA was performed prior to mastectomy. Needles were rinsed in cold PBS-heparin solution and the samples were transported on ice. A 10% aliquot was used to prepare a ThinPrep slide for Papanicolaou staining; another 10% aliquot was reserved for immunohistochemistry, and the remainder was used for RNA extraction (Trizol and RNeasy).

Cytomorphology evaluation was blinded to all participant data and was performed by an expert cytopathologist (CZ) with extensive experience with FNA sample scoring using the Masood score (MS). The components of MS include cellular arrangement, pleomorphism, myoepithelial cells, anisonucleosis, nucleoli, and chromatin clumping. This classification system provides a validated method to classify rFNA samples from unaffected women; categories include non-proliferative (score 6–10), proliferation without atypia (PWA 11–14), and proliferation with atypia (15–18) [16]. We defined atypical samples as MS > 14.

### 2.2. Total RNA extraction and gene expression profiling to identify hormone-related genes

Total RNA was extracted from rFNA samples using Trizol (Invitrogen) and purified using RNeasyPlus Micro Kit (Qiagen). The RNA was then treated with DNase and checked for integrity using Agilent 2100 Bioanalyzer. The median RNA yield was 283.2 ng and median RNA integrity was 7.6. A subset of 30 samples with sufficient RNA for gene expression analysis was selected for gene array as previously described [22]. Total RNA sample labeling and hybridization were performed by Northwestern University Genomics Core Facility using Illumina Human WG6 BeadArray which contains 48,804 50-mers probes to detect >25,400 unique curated genes. The microarray data was deposited on Gene Expression Omnibus (GEO) with accession number GSE36318. The raw data were normalized using the robust spline (RSN) algorithm available in the Lumi Bioconductor package for R statistical software. Non-specific filtering was performed by limiting analyses only to those with interquartile range above the median.

Serum samples acquired on the day of surgery were available from 18 women in the Illumina array subset. Estradiol and progesterone concentration was measured using radioimmunoassay kits (Diagnostic Systems Laboratories). Follicle-stimulating hormone (FSH) concentration was measured using an enzyme immunoassay (Alpco Diagnostics). Genes that demonstrated significant correlation of expression with estradiol, progesterone, FSH concentrations were identified; those with Pearson correlation coefficient $R > 0.60$ indicated highly significant correlation ($P < 0.005$). With the hypothesis that hormone-related gene exposure may contribute to cellular atypia, we chose 20 target genes that were highly correlated to serum estradiol, progesterone, or FSH, or related to menopausal status. These included 5 estrogen-associated genes (PDZK1, CAMK2B, AGT, PIK3R1, FOXL2), 5 progesterone-associated genes (TNFSF11, ITGA1, F3, LCK and RUNX1T1), 5 follicle stimulating hormone (FSH)-associated genes (GPR87, MAPK10, EEF1B2, RPSA and EEF1A1). In addition, we included 5 genes that we have previously reported as being related to premenopausal status in a previous study (CCND1, GREB1, PGR, PRLR and TFF1) [23].

### 2.3. Quantitative RT-PCR

Total RNA (100 ng) of 54 samples was reverse-transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) and pre-amplified using TaqManPreAmp Master Mix (Applied Biosystems). Primers of 20 target genes and two housekeeping genes (GAPDH and HPRT1) were preloaded in 384 well micro fluidic cards (each gene in triplicate) for Taqman low density gene expression assays (TLDA) from Applied Biosystems. Assays were designed with small amplicons (<100 bp) to enhance detection sensitivity. Real Time PCR reactions were carried out in an Applied Biosystems 7900HT machine.

### 2.4. Statistical analysis

For each target gene, expression level was normalized against the average expression of two housekeeping genes. The difference between the two categories was compared using two-tailed t-test with Sidak adjustment for multiple comparison, $P < 0.005$ as statistically significant. Cytologic analyses were performed on the rFNA samples from women enrolled in the study using the Masood score. Linear regression analyses were performed relating each score to recency of pregnancy, stratified for parity and adjusted for age. Clustering analysis was performed using CIMminer from NCI Genomics and bioinformatics Group (http://discover.nci.nih.gov/)