Original Study

Vaginal Gene Expression During Treatment With Aromatase Inhibitors

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Abstract

Vaginal gene expression in aromatase inhibitor-treated women was compared with postmenopausal control women treated with vaginal estrogen therapy. Vaginal tissue from aromatase inhibitor-treated women had low expression of genes involved in cell differentiation, proliferation, and cell adhesion, and associated with vaginal discomfort. The presence of vaginal aromatase suggests that this is the result of local and systemic aromatase inhibition.

Background: Aromatase inhibitor (AI) treatment suppresses estrogen biosynthesis and causes genitourinary symptoms of menopause such as vaginal symptoms, ultimately affecting the quality of life for many postmenopausal women with breast cancer. Thus, the aim of this study was to examine vaginal gene expression in women during treatment with AIs compared with estrogen-treated women. The secondary aim was to study the presence and localization of vaginal aromatase. **Patients and Methods:** Vaginal biopsies were collected from postmenopausal women treated with AIs and from age-matched control women treated with vaginal estrogen therapy. Differential gene expression was studied with the Affymetrix Gene Chip Gene 1.0 ST Array (Affymetrix Inc, Santa Clara, CA) system, Ingenuity pathway analysis, quantitative real-time polymerase chain reaction, and immunohistochemistry. **Results:** The expression of 279 genes differed between the 2 groups; AI-treated women had low expression of genes involved in cell differentiation, proliferation, and cell adhesion. Some differentially expressed genes were found to interact indirectly with the estrogen receptor alpha. In addition, aromatase protein staining was evident in the basal and the intermediate vaginal epithelium layers, and also in stromal cells with a slightly stronger staining intensity found in AI-treated women. **Conclusion:** In this study, we demonstrated that genes involved in cell differentiation, proliferation, and cell adhesion are differentially expressed in AI-treated women. The expression of vaginal aromatase suggests that this could be the result of local and systemic inhibition of aromatase. Our results emphasize the role of estrogen for vaginal cell differentiation and proliferation and future drug candidates should be aimed at improving cell differentiation and proliferation.

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Introduction

Aromatase inhibitor (AI) treatment is used for breast cancer therapy in postmenopausal women, and by inhibition of endogenous estrogen synthesis, the treatment results in extremely suppressed estrogen levels.[1,2] Low estrogen levels, in turn, are associated with genitourinary symptoms of menopause.[3] Vaginal atrophy symptoms and sexual dysfunction are especially problematic for AI-treated women,[4,5] which might affect quality of life.[6] In healthy postmenopausal women, genitourinary symptoms of menopause (eg, vaginal dryness, itching, and pain during intercourse), are usually treated with vaginal estrogen therapy.[6] However, this treatment is not recommended for breast cancer patients treated with AIs.[7,8] Nonhormonal vaginal moisturizers are unfortunately seldom sufficient for long-term effects[9] and new treatment options are warranted.

Aromatase inhibitor treatment results in low vaginal epithelial cell proliferation and altered steroid hormone receptor staining intensity in the vaginal mucosa, which suggests a possible local inhibition of vaginal aromatase in addition to the effects of systemic estrogen deprivation.[10] However, the effect of AIs on vaginal gene expression has not been extensively studied. Vaginal gene expression analysis after systemic hormone replacement therapy[11] and in relation to

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vaginal dryness[12] suggests a downregulation of genes important for cell growth, proliferation, epithelial structure, cell differentiation, and barrier function in estrogen-deprived atrophic vaginal samples.[11,12] The expression of vaginal aromatase mRNA[13,14] and protein in the vagina of cynomolgus monkeys[14] suggests a possible local inhibition of vaginal aromatase during treatment with AIs,[10] which in turn, might have additional effects on gene expression.

Considering that breast cancer is the most common female cancer with an increasing number of women with longer life expectancy, any effect on quality of life during AI treatment has become extremely important. Therefore, studies on the effect of AIs on vaginal tissue are needed to develop effective treatment options for women who suffer from vaginal symptoms, in circumstances in which vaginal estrogen treatment is unsuitable. Thus, with the aim of identifying downstream targets of the estrogen receptor, for potential use in the treatment of vaginal symptoms, in this study we set out to investigate gene expression in vaginal tissue from AI-treated women compared with vaginal tissue from healthy postmenopausal women treated with vaginal estrogen therapy. In addition to the gene expression, a secondary aim was to study the presence and distribution of vaginal aromatase, to further understand the role of vaginal aromatase during vaginal symptoms caused by treatment with AIs.

Patients and Methods

Study Subjects and Design

This study was part of a larger cross-sectional study on urogenital symptoms and sexual function in postmenopausal women with breast cancer receiving adjuvant endocrine treatment and healthy postmenopausal women, and the study subjects have been described.[2,4,5,10,15] This substudy included AI-treated women with breast cancer (n = 28) and postmenopausal control women treated with vaginal estrogen therapy (estradiol or estriol; n = 35). Of these, RNA quality was sufficient for gene expression analysis for 15 AI-treated and 22 control women, and 4 and 5 respectively, were selected for microarray analysis. Women with breast cancer without adjuvant therapy, tamoxifen-treated women, AI-treated women receiving vaginal/systemic estrogen therapy, and control women without vaginal estrogen therapy or with systemic estrogen therapy were excluded for this substudy. Briefly, all women with breast cancer had been treated with AIs for > 6 months and did not have any other ongoing primary cancer therapy, and no recurrent cancer or other severe diseases. Postmenopausal status was defined by at least 12 months of amenorrhea. Control women had been treated with vaginal estrogen therapy for at least 6 months and did not have any previous cancer or serious disease.

All participants attended a previously described health examination.[4,5,10,15] Compliance with AI therapy was requested (never missing/missing once a week/missing once a month). Data were collected on medical history, drug therapy, previous and current adjuvant endocrine therapy, and previous and current hormonal treatment. Weight and height were measured in a standardized manner and body mass index (BMI) calculated.

Hormone Analysis

Blood samples were collected, centrifuged, and stored at −20°C until hormone analysis using liquid chromatography tandem mass spectrometry as previously described.[2,15]

Vaginal Biopsies

Vaginal atrophy was evaluated (classified as none, mild, moderate, or severe) and vaginal pH measured using a paper indicator (Machery-Nagel, Germany). A vaginal punch biopsy was taken from the deep fornix. The biopsies were put in RNA-later (Qiagen, Hilden, Germany) for 24 hours and then stored at −70°C until RNA isolation or fixed in 4% formalin for 24 hours and then stored in 70% ethanol until embedded in paraffin and used for immunohistochemistry.

Total RNA Isolation

Total RNA was isolated from vaginal biopsies using an miRNeasy mini kit (Qiagen, Hilden, Germany) or the TRIzol method (Life Technologies Europe BV, Stockholm, Sweden) according to the manufacturer's instructions.

Microarray Expression Analysis

RNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA). All samples included had an RNA integrity number value > 5. Total RNA (250 ng) from each sample was used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the Ambion WT Expression Kit (P/N 4425209 Rev C 09/2009) and Affymetrix Gene Chip WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev. 5; Affymetrix Inc, Santa Clara, CA). GeneChip ST Arrays (GeneChip Gene 1.0 ST Array) were hybridized for 16 hours in a 45°C incubator, rotated at 60 rpm. According to the GeneChip Expression Wash, Stain and Scan Manual (PN 702731 Rev 3, Affymetrix Inc) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip Scanner 3000 7G.

Microarray Data Analysis

Raw data from the microarray analysis were normalized in the free software Expression Console provided by Affymetrix (*http://www.affymetrix.com*) using the robust multiarray average method.[16,17] Subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (*http://www.r-project.org*) using packages available from the Bioconductor project (*www.bioconductor.org*). To search for the differentially expressed genes between study groups, an empirical Bayes moderated paired t test with robust regression was then applied,[18] using the 'limma' package.[19] To address the problem with multiple testing, the P values were adjusted using the method of Benjamini and Hochberg.[20] Genes with an adjusted P value $< .05$ and an average $\log_2$ fold-change of at least 1 were regarded as differentially expressed.

Bioinformatic Analysis of Differentially Expressed Genes

Cluster analysis and principal component analysis were performed using Genesis version 1.7.6[21] to visualize differentially expressed genes. Functional analysis and gene ontology were studied using Database for Annotation, Visualization, and Integrated Discovery (DAVID) with the whole genome as background. A significant Benjamini P value, corrected for multiple testing and a fold enrichment score limit set to 1.3 according to the software recommendations[22] were studied. Networks of differentially expressed genes were

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