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Original article

Computer-assisted image analysis of breast fine needle aspiration in a randomized chemoprevention trial of fenretinide vs. placebo in HRT users

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Abstract

Background: Digital nuclear morphometric analysis can capture subtle differences along neoplastic progression. Studies showed different profiles from normal to cancer lesions. Our goal is to utilize this method as biomarker in chemoprevention trials.
Methods: Postmenopausal women were randomized to oral (CEE) or transdermal (E2) estrogen replacement therapy (ERT) in association with fenretinide or placebo. Ultrasound-guided fine needle aspiration (FNA) was performed at baseline and after 12 months in a subset of subjects.
Results: Ten samples were analyzed by karyometry. E2 compared with CEE increased nuclear area ($p = 0.01$). A similar pattern was observed for other DNA content and chromatin texture features. Fenretinide vs. placebo, increased nuclear area and shape while decreased slope, peak and entropy.
Conclusion: Preliminary results indicate that nuclear morphometry is feasible on FNA samples. ERT and fenretinide induced significant karyometric changes. These results support further investigation of this procedure as surrogate biomarker in chemoprevention trial.

Keywords: Biomarker; Breast cancer; Chemoprevention; Estrogen replacement therapy; Fenretinide; Karyometry

Introduction

Cancer chemoprevention strategies are rapidly increasing. It is crucial to develop novel intermediate biomarkers (surrogate end-point biomarkers, SEBs) for a better characterization of at-risk populations, a quicker screening of new compounds and drugs efficacy.

Tissue SEBs are more interesting since they may catch alterations directly connected to the pathological progression.[1] Among the different procedures to study cell biomarkers for breast cancer, fine needle aspiration (FNA) is inexpensive and may be repeated over time with a minimum morbidity. Random sampling of the breast gland with FNA is supported by the "field carcinogenesis" theory.[2] This technique allows, in addition to the standard cytology, the assessment of a wide

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range of biomarkers, for example estrogen and progesterone receptors, Ki67, HER2/neu, and survivin.[3–5]

At the DNA level, chromatin organization is emerging as an important epigenetic event that modulates gene transcription involved in tumorigenesis. Abnormal chromatin patterns and methylation status might represent promising SEBs for chemoprevention trials.[6] Computer-assisted nuclear imaging can provide a quantitative useful measurement to characterize early stage of progression and to compare the effect of different treatments.[7]

In an attempt to reduce breast cancer risk associated with hormonal replacement therapy (HRT) we conducted a two-by-two factorial randomized phase II trial. Healthy postmenopausal women were randomized to receive fenretinide (4-HPR), a synthetic vitamin A derivative, or placebo; and oral conjugated equine estrogen (CEE) or transdermal 17-beta estradiol (E2), for 1 year. Fenretinide has shown a breast cancer chemopreventive effect especially in premenopausal women,[8] suggesting a hormonal interaction effect. The original study compared the effects of the treatment on several biomarkers associated with breast cancer risk, including circulating insulin-like growth factor-I (IGF-I), IGF binding protein-3 (IGFBP-3), IGF-I/IGFBP-3 molar ratio, sex-hormone binding protein (SHBG), and mammographic density. The results are reported elsewhere.[9] To further study other possible SEBs, an exploratory analysis was conducted to identify changes in chromatin organization, in breast cells obtained by FNAs, using a computerized cyto-morphometric analysis. The aim of the study was to check whether there was a change in mean karyometry measurements after 1 year of treatment, and whether this change was related to the administered treatment (type of HRT or use of fenretinide).

Subjects and methods

Participants

Study participants were postmenopausal healthy women who were candidates to HRT for menopausal syndrome. The study received IRB approval (study European Institute of Oncology (EIO) #167) and all women gave their signed informed consent. Study design has been described elsewhere.[9] In brief, subjects were randomly assigned by a two-by-two factorial design to oral (CEE) vs. transdermal (E2) continuous sequential HRT, plus fenretinide vs. placebo for 12 months. Sequential medroxyprogesterone acetate (MPA) was added in each arm. Assignment to fenretinide and placebo were blinded, whereas the estrogen route was unblinded, but the pathologist was blinded to all arms.

The study was conducted at four academic institutions in Italy, in the present study we analyzed a subgroup of subjects recruited at the EIO, Milan, Italy.

Histochemical staining

An ultrasound-guided fine needle aspirate of the breast was performed in subjects who agreed to this procedure, using the technique described by Fabian et al.,[10] slightly modified. In brief, fine aspiration with a 22 gauge needle was performed under ultrasound-guide in an area of rich parenchymal component, usually hypoechoic, where the probability of obtaining adequate material is the highest.[11] Tissue was probed deeply in an attempt to sample the terminal ducts, where the epithelial cells thought to give rise to breast cancer are located.[12] The exact site of sampling was recorded in order to repeat the aspirate in the same location after 1 year of intervention. Cytologic material was fixed in ethanol and stained according to the Papanicolaou technique in order to perform a standard cytology. The degree of epithelial dysplasia was assessed using the classification described in the guidelines for non-operative diagnostic procedures and reporting in breast cancer screening.[13]

Each stained cytological slide, containing breast cells, was bleached and stained applying the Feulgen method (Becton Dickinson, San Jose, CA, USA). The staining was performed according to the manufacturer's booklet.

Image analysis

The samples were selected based on the presence of at least 40 nuclei suitable for karyometry both at the baseline and 12-month assessment times.

The morphometric, DNA, and chromatin texture analyses of all cytological slides were carried out using a CAS 200 image analyzer (Becton Dickinson). The Feulgen reaction stripped away all non-nuclear material leaving only blue nuclei. The nuclear DNA content, in the presence of concentrated hydrochloric acid, was hydrolyzed into its constituent nucleic acids. The Feulgen dye then bound stoichiometrically to the nucleic acids. The CAS 200 digitalized the bright-field absorption into a series of pixels that were quantified from optical density readings. The sums of the pixels' optical densities for each nucleus were compared with the summed optical density of rat hepatocyte nuclear DNA, which was used as a standard external control of known diploid DNA content. This allowed conversion of the individual nuclear images into picograms of DNA. Moreover, the different optical density values of all the pixels, which form the digitalized image of each nucleus, were used to calculate the values of all chromatin texture analysis features.

For the measurement of the morphometric, DNA, and chromatin texture features, a board-certified cytotechnologist, specifically trained to perform image analysis, captured the neoplastic nuclei from all cytological slides using a 40× lens. Using routinely accepted standards for nuclear atypia, such as size, shape, and hyperchromasia, the technologist captured all atypical well-preserved nuclei for quantitation. When necessary, the technician, using the instrument's mouse, manually segmented nuclear images. These segmentation steps were conducted at the discretion of the technologist. Nuclear segmentation was performed only when nuclear images "touched" each other and were