Scientific
Research
Publishing

Detection of Estrogen Responsive Breast Cancer Circulating Tumor Cells: Assay Development for Anti-Hormone Therapy Resistance

Sally A. Litherland[1*], Louis Barr[2], Robert Reynolds[2], Elizabeth Griffith[1], Ryan Sause[1], Tiffany Encarnacion[1], Alvin J. O. Almodovar[1], Xiang Zhu[1], Stephanie Dickstein[1], Yai-Ping Shao[1], Na'im Fanaian[3], David A. Decker[1,2]

[1]Translational Research, Florida Hospital Cancer Institute, Orlando, FL, USA
[2]Breast Cancer Oncology, Florida Hospital Cancer Institute, Orlando, FL, USA
[3]Diagnostic Pathology, Florida Hospital Cancer Institute, Orlando, FL, USA
Email: [*]Sally.Litherland@flhosp.org

Abstract

Recent clinical trials with histone deacetylase inhibitors (HDACi) have shown increased progression free survival by re-sensitizing resistant estrogen receptor positive (ER+) breast cancer cells to hormone suppressive therapies (HT). However, these trials lacked a sensitive, specific assay to identify and monitor HDACi/HT sensitive or resistant tumors. We tested detection of ER expression and histone acetylation of chromatin at the growth regulation by estrogen in breast cancer 1 (*GREB*1) gene, an estrogen-responsive gene involved in ER expression, in circulating tumor cell (CTC) as potential candidate assays for HDACi/HT sensitivity. ER+ and ER− CTC were detected and isolated from breast cancer patient peripheral blood by high speed fluorescence activated cell sorting (FACS) for use in mRNA analysis and anti-acetylated histone-mediated Chromatin Immunoprecipitation (ChIP). cDNA from mRNA and DNA extracted from the ChIP isolates were quantified by real-time PCR for *GREB*1. CTC isolates from patients who had an ER+ breast cancer primary contained both ER+ and ER− cells. More ER+ than ER− CTC was found in HT sensitive patients compared to HT resistant patients (p = 0.0559). *GREB*1 was found in acetylated histone chromatin from both ER+ and ER− CTC. The number of ER+ and ER− CTC found in peripheral blood appears to parallel patient outcomes as to their sensitivity to HT. Acetylated histone analysis can detect chromatin containing *GREB*1 in CTC, suggesting it may be useful as a more specific measure of HDACi effects on breast tumor cells. A larger, longitudinal data set following patients through HT/HDACi trials is needed to confirm these observations and their development for clinical use.

---

[*]Corresponding author.

Keywords

Epigenetics, Breast Cancer, Estrogen Receptor, Histone Acetylation, Anti-Hormone Therapy

1. Introduction

Breast cancer incidence in the United States is currently reported by the National Institutes for Health as between 63.51 to 98.69 cases per 100,000 among women, with a marked decline in mortality in the last decade [1]. Great advancements have been made in early detection and treatment of primary breast cancer through greater awareness of the value of regular prognostic screening, and advancements in surgical, chemotherapeutic, hormonal, and radiation therapies. However, metastatic manifestations of breast cancer have proven elusive to early detection and devastatingly retractable to cure. Early detection and efficient monitoring of metastasis could mean the difference between recovering quality of life and severely debilitating terminal illness. Unfortunately, once tumor cells enter the circulation, they can de-differentiate into stem cell-like morphologies conducive to metastatic invasion [2]. This means their character changes, often to the point of becoming resistant to original treatment given to a patient.

Approximately 70% of breast cancers are identified as estrogen receptor positive (ER+), indicating that the tumor can grow in response to estrogen or estrogen-like hormones [3]. Current treatment for ER+ cancers for women who are pre-menopausal includes estrogen receptor modulating drugs such as Tamoxifen and estrogen receptor antagonists such as Valoxifene and Faslodex. For post-menopausal women or women who have had their ovaries removed, anti-aromatase inhibitor therapy is used to block androgen conversion to estrogen, especially in adipose tissue [3]-[5]. Paradoxically, estrogen deprivation meant to block tumor growth can increase the responsiveness of the tumor to estrogen-like molecules, making effective hormone treatment a concern for breast cancer survivors [4] [5]. Moreover, growth of tumors in estrogen rich environs can promote ER expression and lack of the hormone may promote rise of ER− cells which can enter the circulation and pose a relapse or metastatic potential risk [5]. There is little data on the rate of ER+ to ER− conversion in tumor cells and the impact of hormone blockade therapies on this process. Therefore, for optimal hormonal therapy to be beneficial to breast cancer survivors, it is necessary to monitor their tumor status over time and treatment.

It is still not known if tumor cells develop their metastatic potential and unique characteristics in the primary tumor or in response to components of blood [6]. Discreet or individual tumor cells in the body's tissues are not easily identified by current imaging techniques. Only when tumor cells enter the circulation as discreet single cells is there the chance to capture their new characteristics and evaluate their possible fates in what is now being called a "liquid biopsy" [7] [8]. Therefore, detecting and monitoring of circulating tumor cells (CTC) in a patient as they evolve and change from before primary treatment all the way through post-surgical and maintenance treatment may be key to providing the most effective treatment for each individual and each relapse. CTC analysis has recently emerged from research use to strong clinical potential with the 2004 FDA approval of CTC counts to monitor relapse/release of tumor cells after primary treatment [9]-[14].

Early in breast cancer oncology, the discovery of breast cancer growth stimulation through ovarian hormones indicated the expression of estrogen receptors (ER) by tumor cells as a target for directed anti-hormone therapies. Direct antagonists of the ER and indirect depletion of circulating sources of estrogen (oophorectomy and anti-aromatase inhibitor (AI) therapies) became strong therapies for controlling ER mediated tumor growth [15]. Numerous selective estrogen receptor modulator therapies have been proposed and tested for use in blocking ER function in pre-menopausal women as single therapies or in hormone combination with mixed success [15]-[17]. In post-menopausal and oophorectomized women blockade of adipose production of estrogen from androgens is most often accomplished using long term adjuvant HT therapies. A problem arising from chronic exposure to anti-hormone therapies is the unintentional selection of estrogen resistant cells within the tumor population, either by de-sensitization of the ER or by causing ER downregulation and estrogen independent growth of ER− cells arising from the original ER+ tumor [18].

HT resistant and estrogen independent breast cancer cells can seed tumor relapse or metastasis causing failure of the HT therapy. As research progresses into the role of hormone stimulation in tumor growth and metabolism, a number of potential therapeutic targets have emerged including signal transduction molecules, gene targets

with ER response elements, and estrogen responsive gene regulatory molecules such as microRNA and epigenetic modification [19]-[22]. To prevent catastrophic treatment failure, combination therapies are being put to clinical trial, most directed at suppression of the emerging treatment resistant subpopulations. A second approach is being tested using agents that will augment the original anti-hormone therapy by increasing the expression and sensitivity of ER proteins on these tumor cells, thus improving the effectiveness of the primary anti-hormone therapies. One class of combination therapy drugs has shown promise in recent trials: inhibitors of histone deacetylases (HDAC) [16] [20]-[22]. HDAC enzymes remove acetyl groups from epigenetically modified histones, allowing for re-formation of the histone nucleosome core which "closes" chromatin to access by DNA modulating enzymes that transcribe or manipulate the chromatin for dynamic functions such as gene expression, recombination, replication and mutation [23]. Inhibiting HDAC allows for prolonged expression of genes including the ER itself, increasing the sensitivity of the cell to ER signaling, increasing the rate of fatal mutation and cell death in estrogen deprivation conditions such as induced by effective HT [20]-[22].

A recent Phase II clinical trial study of combined HT and HDACi treatments Exemestane and Entinostat showed potential for a subset of HT resistant patients with high general acetylation of this combined therapy regimen reversing HT resistance and prolonged progression free survival for 4 to 6 months more than Exemestane treatment alone [22]. This study also brought to light a major drawback seen in HDACi/HT clinical trials: the lack of a sensitive and clinically selective monitoring assay for the effectiveness of the combination treatment. The use of acetylated proteins in blood was noted to be at best an imprecise estimate of HDAC activity in tumor cells and contributed ambiguity to an otherwise promising new therapeutic option. Without a direct measure of anti-HDAC treatment affecting ER signaling in tumor cells, it is difficult to correlate the treatment with re-sensitizing cells to HT treatment. This would hinder dose response studies and preclude measurement of histone acetylation and/or HDAC activity as a potential predictive measure for HT resistance susceptibility.

Circulating Tumor Cell (CTC) phenotyping and quantification have recently become of great interest in diagnostic and treatment efficacy monitoring protocols for cancer patients at risk for relapse or metastasis [6] [24] [25]. The majority of work in this area has concentrated on quantitation of tumor cell numbers in blood using fixed cell samples analyzed microscopically for phenotypic changes to a more "stem cell-like" character indicative of metastatic potential [10] [26]-[29]. In this study, we aseptically collect live CTC from the peripheral blood of breast cancer patients with known ER+ metastatic breast cancer to analyze histone acetylation of *GREB*1, a gene expressed in response to estrogen and involved in regulation of ER in breast and breast cancer cells [19] [20], to determine if such estrogen-responsive gene activation indicator analyses could afford a better, more specific monitoring assay for following the mechanism and efficacy of HDACi treatment in re-sensitizing HT resistant breast tumor cells and promoting better efficacy of adjuvant HT treatment in preventing relapse and metastatic recurrence of ER+ tumors.

2. Materials & Methods

2.1. Pilot Study Population and Sample Collection

Table 1 outlines the demographics of the pilot study population. Table 2 describes the inclusion/exclusion criteria for participation for breast cancer patients and healthy controls. Fourteen cancer patients and 2 healthy control participants were consented for this study under Institutional Review Board (IRB) approved protocols (IRBNet # 372522/583244/583243). At the consenting visit, each participant completed a questionnaire regarding medical history and arrangements were made for the blood collection. At their next study visit, consented participants were asked to donate 2 tubes (approximately 20 ml) of blood via venipuncture for the study. Blood was drawn in an approved blood collection setting by certified phlebotomist research staff or clinical personnel into heparinized vacutainers and immediately labeled with study specific code identifier. Participants' clinical progress was followed for up to 1 year through audits of their clinical records to monitor progression free survival. Data collected from medical records and analytical testing was Health Information Portability and Accessibility Act (HIPAA) protected and anonymized for inclusion in the reportable study data set and analyses.

2.2. Sample Processing for CTC Isolation

For CTC isolation, patient blood samples were separated on ficoll histopaque gradients to isolate peripheral blood mononuclear cells (PBMC) and washed into rich growth medium (RPMI 1640 (Mediatech Manassas, VA),

Table 1. Study subject population parameters.

Patient Group	(N)	Sample Analyzed[*]	Age in years (mean ± SD)	Metastatic Cancer	BMI (mean ± SD)	HT[**]
ER+ HT sensitive	5	4/5[*]	66.6 ± 5.6	4/4	24.4 ± 3.5	multiple
ER+ HT resistant	2	2/2	65.0 ± 1.4	2/2	29.5 ± 2.1	multiple
ER− primary, metastatic	1	1/1	27	1/1	28.0	Neo-adjuvant
ER+ primary	4	2/4[*]	56.8 ± 11.2	0/2	35.0 ± 14.1	None
Healthy controls	2	2/2	56.0 ± 5.7	0/2	26.5 ± 9.2	None

[*]Samples not analyzed due to screen failure and/or lost to follow-up after consent. [**]HT = Hormone suppressive therapies

Table 2. Participant inclusion/exclusion criteria.

Inclusion Criteria for Patients Women (>18 yr old) who have survived primary breast cancer (with surgery, neo-adjuvant, adjuvant and/or radiation therapy) and are now presenting with either relapse or metastatic disease; <u>or</u> who presented with primary breast cancer and treated by mastectomy followed by chemotherapy and/or aromatase inhibitor therapy.	**Exclusion Criteria for Patients** Men Pre- or post-menopausal women without a personal history of breast cancer, Women with untreated primary tumor breast cancer patients or did not going to undergo treatment at FHCI, Individuals deemed not healthy enough to safely participate in the study by the study associated physicians.
Inclusion Criteria for Controls Women >18 yr of age, who have never had and do not currently have breast cancer.	**Exclusion Criteria for Controls** Men, Pre- or post-menopausal women with a personal history of breast cancer, Individuals deemed not healthy enough to safely participate in the study by the study associated physicians.

10% medium 199 (Gibco, Life Technologies, Grand Island, NY), 10% fetal calf serum (Mediatech), 2% antibiotic-antimycotic mix (Sigma-Aldrich, St Louis, MO) for fluorescence-activated cell sorting (FACS) collection. The PBMC samples were labeled with fluorescently conjugated antibodies specific for EPCAM, CD45, Met-1, EGFR, and ER proteins (BD Biosciences (San Jose, CA), Beckman Coulter (Miami, FL); Millipore (Billerica, MA)). The labeled samples were then subjected to flow cytometric identification and FACS separation in a sterile high speed collection using the Florida Hospital Cancer Institute custom-built MoFlo MDX Sorter instrument (Beckman Coulter) housed in a Class 2A laminar flow safety cabinet (Walker, UK). The sorted cell populations were collected into the same rich culture medium and further processed for CTC isolation and molecular analysis.

2.3. Chromatin Acetylation Modification ChIP Analysis

ChIP analysis [30] on *ex vivo* CTC includes precipitations with anti-acetylated histone H3 modification specific antibodies (Millipore), in parallel with non-specific IgG and total DNA controls. ChIP isolates captured crosslinked DNA fragments associated with these epigenetic modification and expression molecules at the time of fixation. DNA fragments were sonicated to approximate 1000 bp lengths and isolated by rapid DNA purification/ethanol precipitation. ChIP isolated DNA were subjected to relative quantitation using realtime PCR with TaqMan (Life Technologies, Grand Island, NY) primers for *GREB*1, *ERalpha,* and *GAPDH*. Association of *GREB*1 and *ERalpha* gene fragments with acetylated histone was considered indicative of epigenetic regulation of expression at these gene sites. Relative expression levels expressed as R values = ln 2ΔΔCT [31] were analyzed at cycles within the linear phase of the PCR and quantitated relative to internal standard and housekeeping *GAPDH* gene expression in the same sample.

2.4. Protein Expression Analysis

Expression of cell surface and intracellular *ERalpha* and *GREB*1 was quantified using CTC flow cytometric analysis of cell surface staining. The relative expression of *ERalpha* was compared between HT resistant and

HT sensitive ER+ and ER− sorted CTC.

2.5. RNA Expression Assay

mRNA was isolated from sorted ER+/ER− CTC using RNALater (Ambion, Life Technologies, Grand Island, NY) fixation and Trizol extraction (Ambion). The mRNA was reverse transcribed to cDNA and then amplified using reverse transcriptase-realtime polymerase chain reaction (RT-realtime PCR; Qiagen, Valencia, CA) protocols. The RT reaction was run with oligo dT and random primers to generate cDNA from mRNA by action of reverse transcriptase (2 hr, 37 C), followed by RT inactivation at 85 C for 5 minutes. PCR Taq polymerase amplification was done with a 10 min hot start at 95 C followed by 40 cycles of 95 C 1 min, 55 C 1 min (internal sequence data collection at end), 72 C 1 min extension, using commercially available TaqMan gene-specific primers, fluorescently labelled internal sequence reporter, and internal ROX dye control (Life Technologies). Relative expression levels expressed as Ln R value $= 2^{(GAPDH\ \Delta Ct) - (GREB1\Delta Ct)}$, as analyzed at cycles within the linear phase of the PCR and quantitated relative to internal standard and housekeeping *GAPDH* mRNA expression in the same sample.

2.6. Plasma Estradiol Assay

Plasma samples were collected from the top of the ficoll histopaque gradient separation of PBMC and frozen at −80 C. Samples were later thawed and immediately used in a commercially available competitive sandwich enzyme enzyme-linked immunosorbant assay (ELISA) analysis optimized for detection of estradiol in biological fluids (Oxford, Rochester Hills, MI). In the quantitative ELISA, capture of estradiol in the sample used anti-estradiol antibodies. Quantitative detection was visualized by competition of estradiol-horseradish peroxidase conjugates added to the ELISA plate with estradiol in the sample inducing a color change in colorimetric substrate (3, 3', 5, 5' tetramethlybenzidine (TMB)) in the presence of hydrogen peroxide. The color change was measured at 650nm using a 96-well ELISA plate reader spectrophotometer.

2.7. Outcomes Criteria for Correlation Analyses

The outcomes to be measured for correlation and comparison includes: Primary breast cancer diagnosis including %ER+ cells by immunohistochemistry, HT & other treatment history, Relapse/metastatic tumor growth history, age, menopausal status, Medical history/demographic information, Progression Free Survival while on HT, and indicators of loss of HT sensitivity/emergence of HT resistance. Data were analyzed using linear regression analysis and compared using multi-factorial (ANOVA) or pair-wise analyses to determine the significance of estrogen-like responses relative to the estradiol and untreated controls using GraphPad Prism 6 Software. Results were considered significant at $p \leq 0.05$.

3. Results

3.1. Estradiol Levels in Plasma Reflect the HT Status of the Patient

As expected, plasma estradiol levels were lower in patients on HT (anti-aromatase therapies) than in patients not on HT and commensurate with the levels seen in post-menopause healthy controls (Figure 1(a)). Of interest, higher estradiol levels were found in patients resistant to HT and/or having ER− CTC numbers greater or equal to ER+ CTC numbers compared with patients who had predominantly ER+ CTC and remained sensitive to HT (Figure 1(b)). These data suggest that ER+ cell populations in HT resistant patients may have access to HT resistant estrogen sources.

3.2. CTC Analyses Reflected the Evolution of ER− Tumor Subpopulations in ER+ Tumor Patients

We found that both ER+ and ER− viable CTC were detectable in all immunophenotypically ER+ tumor patients and the one surgical patient found to have an ER− primary tumor (Figure 2). The ratio of ER+ and ER− CTC reflected the HT sensitivity status of these individuals. ER+ CTC were found highest in HT sensitive individuals (p = 0.055). In all individuals who had progressed on at least on HT agent and were considered clinically HT resistant, higher numbers of ER− CTC were found compared to ER+ CTC.
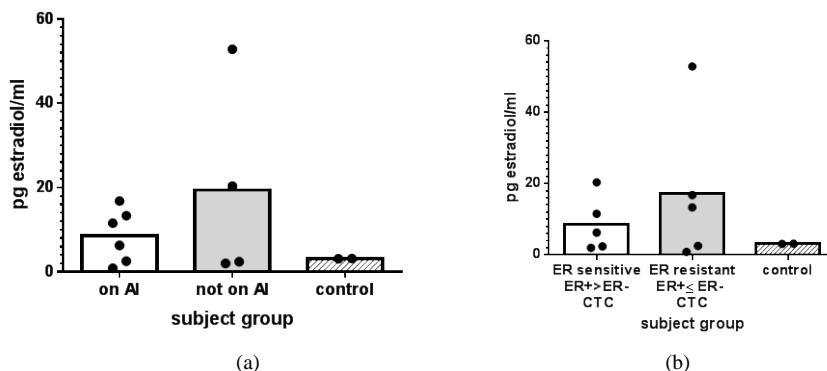
(a)   (b)

Figure 1. Plasma Estradiol Level Comparisons between (a) Patients on HT and those not, and (b) Patients with ER+ > ER− and with ER+ ≤ ER− CTC. When peripheral blood samples used for CTC isolation were separated on ficoll histopaque gradients, plasma was collected from the top of the gradient and frozen at −80 C. The plasma samples were analyzed in triplicate for estradiol by ELISA and reported as mean picogram estradiol per milliliter of plasma. (a) Comparison of plasma estradiol in blood of patients on aromatase inhibitor therapy (AI, n = 6; mean 8.59 ± 6.32) with those not currently on AI (n = 4; mean 19.42 ± 23.89) and post-menopausal healthy controls (n = 2; mean 3.15 ± 0.02). Though a trend for higher estradiol levels was seen in subjects not on AI therapy, no significant difference was found between the groups and all samples had estradiol levels that were within the range of normal peri- or post-menopausal women. (b) Comparison of plasma estradiol in blood of patients deemed ER sensitive (n = 5) or ER resistant based (n = 5) on their HT history and the proportion of ER+ CTC compared to ER− CTC found in their blood. A trend for higher estradiol levels was seen in patients where ER− CTC numbers were greater or equal to that of ER+ CTC, though no statistical significance was obtained in the small dataset.
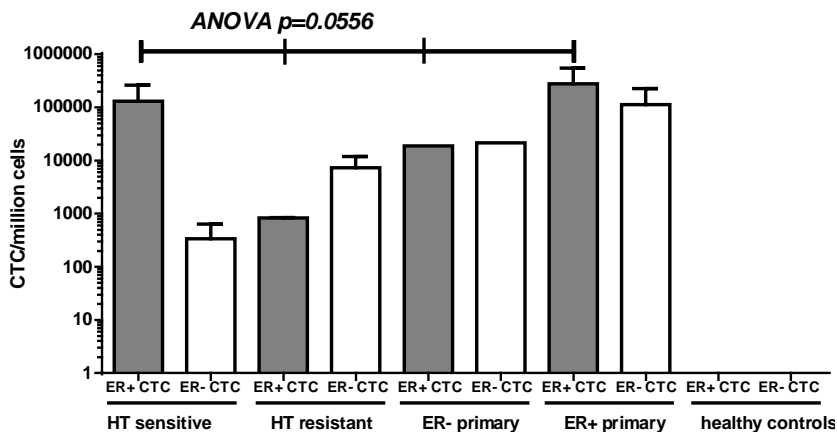

Figure 2. Circulating Tumor Cells in Peripheral Blood: Conversion of ER+ CTC to ER− CTCViable CTC were aseptically collected by high speed FACS using an immunological profile of EPCAM+, Met+, CD45-, and EGFR+/−. CTC were sorted based on detection of the profile and *ERalpha* expression (ER+/ER−). The 2 sorted CTC population counts were standardized to one million cells counted and sorted. CTC Counts of ER+ CTC were compared between AI sensitive and AI resistant subject population and analyzed by ANOVA found to be near but not reaching statistical significance in this small pilot group (p = 0.0559). No statistical significant difference was seen between the CTC counts of ER− CTC compared between AI sensitive and AI resistant subject populations.

3.3. *GREB*1 Gene Regulation by Histone Acetylation in Both ER+ and ER− CTC

Due to the low number of CTC collected in most patients, the limits of ChIP/PCR analysis detection of acetylated histone associated with the *GREB*1 gene locus were limited to samples with >5000 CTC/10^7 cells, namely

samples from blood collected during primary breast tumor excision. Though not enough samples were analyzable to reach statistical significance, the analysis of blood from primary surgical patients show that *GREB*1 loci acetylation is found in both ER+ and ER− CTC (**Figure 3**). *ERalpha* gene analysis did not show any association with histone acetylated chromatin. mRNA analysis of *GAPDH* indicated mRNA was detectable in CTC, but no detection of *GREB*1 and *ERalpha* mRNA was found in the CTC samples. This may be due to the detection limitation of the quantitative RT-PCR analysis and the low CTC numbers analyzed for each sample.

4. Discussion

Since a significant portion of all breast cancers are found to express estrogen receptors, hormone suppressive therapies blocking the production of estrogens or access to its receptor have been strong tools in the oncologist arsenal for post primary breast cancer adjuvant HT treatment [15]. As patients survive longer after primary treatments, the long term efficacy of adjuvant HT therapies deceases by therapy induced-selection of HT-resistant cell subpopulations emerging or developing over time [32] [33].

Clinical trials such as ECOG E2112 are investigating a histone acetylase inhibitor therapy in combination with standard endocrine therapy to overcome hormone resistance. A drawback for HDACi clinical trials in the past has been the lack of a sensitive assay to identify tumor specific HDACi effects contributing to hormone resistance [32].

Trials using HDAC inhibitors with either anti-aromatase inhibitors or selective estrogen receptor modulator treatments have shown meaningful improvement in progression free survival [22] [34] [35]. Entinostat given with Exemestane extended PFS from an average of 2.3 months to 4.3 months in the total tested population but in a subset of patients with high general acetylation, the effective increase was up to 7.3 months progression free survival [22]. As new HDACi treatments emerge for leukemias, lymphomas, and other cancers, it is logical to consider their potential benefit to breast cancer patients because of similar hormone/cytokine responsive characteristics in these cancer cell origin types [19] [22] [32] [33] [36] [37]. Though HDACi therapy has not progressed far enough yet to be a tumor cytotoxic specific agent for therapies, this class of chemotherapeutics has definite possibilities in combined therapy applications. In combined therapies, the strong metabolic toxicity properties of these agents can be diluted and directed for a specific stage or purpose in treatment. Augmentation of HT as a cell sensitizer or re-sensitizer through actions on estrogen sensitivity or through increasing the direct target, aromatase, sensitivity to the primary therapy is an excellent example of such a directed use for HDAC inhibition.
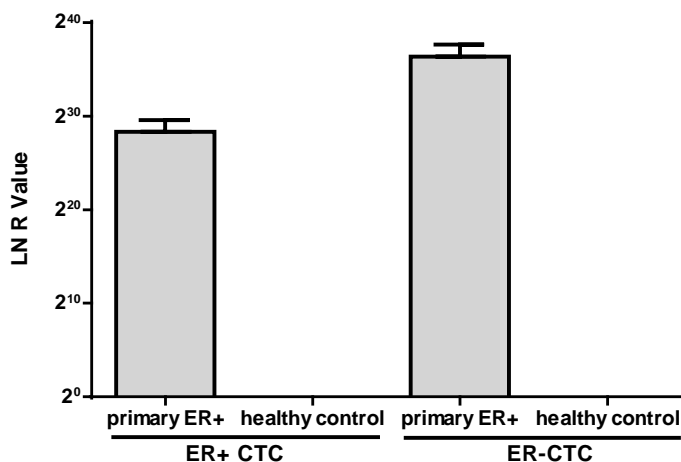

Figure 3. Histone Acetylation on Chromatin Containing Estrogen Responsive *GREB*1 gene Viable CTC collected as described in **Figure 2** were subjected to ChIP isolation of chromatin associated with acetylated histone H3 and amplified by quantitative PCR using primers specific for *GREB*1. ChIP DNA samples from all but the subjects with surgical blood samples taken when their ER+ primary tumors were excised, were too low to accurately quantify. In the surgical blood samples, CTC counts were high enough to quantify the presence of *GREB*1 and *GAPDH* DNA in the samples and identify *GREB*1 DNA in ChIP using anti-acetylated histone H3 antibodies in both ER+ and ER− CTC derived from the blood samples. Relative levels are expressed as Ln R value $= 2^{(GAPDH\,\Delta Ct)-(GREB1\Delta Ct)}$.

Toxicity and specificity are the major cautionary components preventing widespread use of these drugs in therapy trials and regimens [38]. Lack of a measure of true specific mechanism and target of their chemotherapeutic action further delays their acceptance for standard of care clinical use [22]. Therefore, the results of this pilot study could have immense impact on clinical practice providing the needed evidence for direct and specific mechanism of action for a new class of chemotherapeutic treatment sensitizers to prevent therapy failure and emergence of treatment-induced resistance in cancer relapse and metastasis. Such a new treatment option could prevent or at least delay chronic cancer survival from turning into terminal metastatic disease.

Hormone resistance can emerge by a number of molecular mechanisms, many dependent on the continued expression of an estrogen sensitive receptor and a constant requirement of the cells for estrogen to stimulate and sustain growth [22] [23] [36]. In this pilot study, we were able to test ER expression and histone acetylation at the *GREB*1 gene locus, a gene expressed in response to estrogen and involved in regulation of ER in breast and breast cancer cells [19] [20], in both ER+ and ER− CTC of breast cancer patients. Comparing ER+ with ER− CTC in peripheral blood, the ratio of ER+ and ER− CTC appears to parallel with patient outcomes as to their sensitivity or development of resistance to HT. A larger data set and longitudinal follow up of patients through HT treatments is needed to confirm this observation and develop it into a prognostic indicator for clinical use.

5. Conclusion

We present a small pilot study indicating CTC changes seen in ER expression in HT resistant and HT sensitive breast cancer patients during HT treatment. Though the sample size is not large enough to indicate statistical significance in the *GREB*1 epigenetic biomarker analysis, the study results show the feasibility of detecting such epigenetic changes in estrogen responsive genes in CTC found in the peripheral blood of breast cancer patients. Increasing assay sensitivity with larger samples or culture expansion of CTC cells *in vitro* can allow the use of molecular assessment of epigenetic acetylation at ER and ER signal responsive genes such as *GREB*1. This could provide a better, more specific monitoring assay for following the mechanism and efficacy of HDACi treatment in re-sensitizing HT resistant breast tumor cells and promoting better efficacy of HT adjuvant HT treatment in preventing relapse and metastatic recurrence of ER+ tumors. Further development of these analysis measures may yield a precise and specific measure for tracking and potentially predicting the development of hormone therapy resistance in routine blood sampling prior to detectable metastatic growth on imaging. Such a low cost, reliable and minimally invasive way to monitor and possibly preempt treatment failure would have dramatic impact on extending and optimizing the effective use of hormone therapies.

Acknowledgements

Conflict of Interest Declaration

The authors have no financial conflict of interest to report for this work.

References

[1]   Kohler, B.A., Sherman, R.L., Howlader, N., Jemal, A., Ryerson, A.B., Henry, K.A., Boscoe, F.P., Cronin, K.A., Lake, A., Noone, A.-M., Henley, S.J., Eherman, C.R., Anderson, R.N. and Penberthy, L. (2015) Annual Report to the Nation on the Status of Cancer, 1975-2011, Featuring Incidence of Breast Cancer Subtypes by Race/Ethnicity, Poverty, and State. *Journal of the National Cancer Institute*, **100**, 1672-1694.

[2]   Wang, N., Shi, L., Li, H., Hu, Y., Du, W., Liu, W., Zheng, J., Huang, S. and Qu, X. (2012) Detection of Circulating Tumor Cells and Tumor Stem Cells in Patients with Breast Cancer by Using Flow Cytometry. *Tumor Biology*, **33**, 561-569. http://dx.doi.org/10.1007/s13277-011-0303-1

[3]   Renoir, J.-M. (2012) Review: Estradiol Receptors in Breast Cancer Cells: Associated Co-Factors as Targets for New Therapeutic Approaches. *Steriods*, **77**, 1249-1261. http://dx.doi.org/10.1016/j.steroids.2012.07.019

[4]   Iyer, V., Klebba, I., McCready, J., Arendt, L.M., Betancur-Boissel, M., Wu, M.-F., Zhang, X., Lewis, M.T. and Kuperwasser, C. (2012) Estrogen Promotes ER-Negative Tumor Growth and Angiogenesis through Mobilization of Bone Marrow-Derived Monocytes. *Cancer Research*, **72**, 2705-2713. http://dx.doi.org/10.1158/0008-5472.CAN-11-3287

[5] Aktas, B., Muller, V., Tewes, M., Zeitz, J., Kasimir-Bauer, S., Loehberg, C.R., Rack, B., Schneeweiss, A. and Fehm, T. (2011) Comparison of Estrogen and Progesterone Receptor Status of Circulating Tumor Cells and the Primary Tumor in Metastatic Breast Cancer Patients. *Gynecologic Oncology*, **122**, 356-360. http://dx.doi.org/10.1016/j.ygyno.2011.04.039

[6] Matrone, M.A., Whipple, R.A., Balzer, E.M. and Martin, S.S. (2010) Microtentacles tip the Balance of Cytoskeletal Forces in Circulating Tumor Cells. *Cancer Research*, **70**, 7737-7741. http://dx.doi.org/10.1158/0008-5472.CAN-10-1569

[7] Dong, X., Alpaugh, R.K. and Cristofanilli, M. (2012) Circulating Tumor Cells (CTCs) in Breast Cancer: A Diagnostic Tool for Prognosis and Molecular Analysis. *Chinese Journal of Cancer Research*, **24**, 388-398. http://dx.doi.org/10.1007/s11670-012-0279-4

[8] Gioradano, A., Egleston, B.L., Hajage, D., Bland, J., Hortobagyl, G.N., Reuben, J.M., Pierga, J.Y., Cristofanilli, M. and Bidard, F.C. (2013) Establishment and Validation of Circulating Tumor Cell-Based Prognostic Nomograms in First-Line Metastatic Breast Cancer Patients. *Clinical Cancer Research*, **19**, 1596-1602.

[9] Sun, Y.-F., Yang, X.-R., Zhou, J., Qiu, S.-J., Fan, J. and Xu, Y. (2011) Circulating Tumor Cells: Advances in Detection Methods, Biological Issues and Clinical Relevance. *Journal of Cancer Research and Clinical Oncology*, **137**, 1151-1173. http://dx.doi.org/10.1007/s00432-011-0988-y

[10] Pierga, J.-Y., Hajage, D., Bachelot, T., Delaloge, S., Brain, E., Compone, M., Dieras, V., Rolland, E., Mignot, L., Mthlot, C. and Bidard, F.-C. (2012) High Independent Prognostic and Predictive Value of Circulating Tumor Cells Compared with Serum Tumor Markers in a Large Prospective Trial in First-Line Chemotherapy for Metastatic Breast Cancer Patients. *Annals of Oncology*, **23**, 618-624. http://dx.doi.org/10.1093/annonc/mdr263

[11] Riethdorf, S., Fritsche, H., Muller, V., Rau, T., Schindbeck, C., Rack, B., Janni, W., Coith, C., Beck, K., Janicke, F., Jackson, S., Gomet, T., Cristofanilli, M. and Pantel, K. (2007) Detection of Circulating Tumor Cells in Peripheral Blood of Patients with Metastatic Breast Cancer: A Validation Study of the CellSearch System. *Clinical Cancer Research*, **13**, 920-928. http://dx.doi.org/10.1158/1078-0432.CCR-06-1695

[12] Cristofanilli, M., Budd, G.T., Ellis, M.J., Stopeck, A., Matera, J., Miller, M.C., Reuben, J.M., Doyle, G.V., Allard, W.J., Terstappen, L.W.M.M. and Hayes, D.F. (2004) Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer. *New England Journal of Medicine*, **351**, 781-791. http://dx.doi.org/10.1056/NEJMoa040766

[13] Cristofanilli, M., Hayes, D.F., Budd, G.T., Ellis, J.M., Stopeck, A., Reuben, J.M., Doyle, G.V., Matera, J., Allard, W.J., Miller, M.C., Fritsche, H.A., Hortobagyl, G.N. and Terstappen, L.W.M.M. (2005) Circulating Tumor Cells: A Novel Prognostic Factor for Newly Diagnosed Metastatic Breast Cancer. *Journal of Clinical Oncology*, **23**, 1420-1430. http://dx.doi.org/10.1200/JCO.2005.08.140

[14] Pantel, K. (2009) Pathology: Are Circulating Tumor Cells Predictive of Overall Survival? *Nature Reviews Clinical Oncology*, **6**, 190-191. http://dx.doi.org/10.1038/nrclinonc.2009.23

[15] Williams, C. and Lin, C-Y. (2013) Oestrogen Receptors in Breast Cancer: Basic Mechanisms and Clinical Implications. *Ecancermedicalscience*, **7**, 370-382.

[16] Munster, P.N., Thurn, K.T., Thomas, S., Raha, P., Lacevic, M., Miller, A., Melisko, M., Ismail-Khan, R., Rugo, H., Moasser, M. and Minton, S.E. (2011) A Phase II Study of the Histone Deacetylase Inhibitor Vorinstat Combined with Tamoxifen for the Treatment of Patients with Hormone Therapy-Resistant Breast Cancer. *British Journal of Cancer*, **104**, 1828-1835. http://dx.doi.org/10.1038/bjc.2011.156

[17] Shen, C., Huang, Y., Liu, Y., Wang, G., Zhao, Y., Wang, Z., Ten, M., Wang, Y., Flockhart, D.A., Skaar, T.C., Yan, P., Nephew, K.P., Huang, T.H.M. and Li, L. (2011) A Modulated Empirical Bayes Model for Identifying Topological and Temporal Estrogen Receptor Alpha Regulatory Networks in Breast Cancer. *BMC Systems Biology*, **5**, 67-83. http://dx.doi.org/10.1186/1752-0509-5-67

[18] Kubo, M., Kanaya, N., Petrossian, K., Ye, J., Warden, C., Liu, Z., Nishimura, R., Osako, T., Okido, M., Shimada, K., Takahashi, M., Chu, P., Yuan, Y.-C. and Chen, S. (2013) Inhibition of the Proliferation of Acquired Aromatase Inhibitor-Resistant Breast Cancer Cells by Histone Deacetylase Inhibitor LBH589 (Panobinostat). *Breast Cancer Research and Treatment*, **137**, 93-107. http://dx.doi.org/10.1007/s10549-012-2332-x

[19] Rae, J.M., Johnson, M.D., Scheys, J.O., Cordero, K.E., Larios, J.M. and Lippman, M.E. (2005) GREB1 Is Critical Regulator of Hormone Dependent Breast Cancer Growth. *Breast Cancer Research and Treatment*, **92**, 141-149.

[20] Mohammed, H., D'Santos, C., Serandour, A.A., Ali, H.R., Brown, G.D., Atkins, A., Rueda, O.M., Holmes, K.A., Theodorou, V., Robinson, J.L.L., Zwart, W., Saadi, A., Ross-Innes, C.A., Chin, S.-F., Menon, S., Stingl, J., Palmieri, C., Caldas, C. and Carroll, J.S. (2013) Endogenous Purification Reveals GREB1 as a Key Estrogen Receptor Regulatory Factor. *Cell Reports*, **3**, 342-349. http://dx.doi.org/10.1016/j.celrep.2013.01.010

[21] Meng, Q., Chen, X., Sun, L., Zhao, C., Sui, G. and Cai, L. (2011) Carbamazepine Promotes Her-2 Protein Degradation in Breast Cancer Cells by Modulating HDAC6 Activity and Acetylation. *Molecular and Cellular Biochemistry*, **348**, 165-171. http://dx.doi.org/10.1007/s11010-010-0651-y

[22] Yardley, D.A., Ismail-Khan, R.R., Melichar, B., Lichinitser, M., Munster, P.N., Klein, P.M., Cruickshank, S., Miller, K.D., Lee, M.J. and Trepel, J.B. (2013) Randomized Phase II, Double-blind, Placebo-Controlled Study of Exemestane with or without Entinostat in Postmenopausal Women with Locally Recurrent or Metastatic Estrogen Receptor-Positive Breast Cancer Progressing on Treatment with a Nonsteroidal Aromatase Inhibitor. *Journal of Clinical Oncology*, **31**, 2128-2135. http://dx.doi.org/10.1200/JCO.2012.43.7251

[23] Liu, L., Li, Y. and Tollefsbol, T.O. (2008) Gene-Environment Interactions and Epigenetic Basis of Human Diseases. *Current Issues in Molecular Biology*, **10**, 25-36.

[24] Spassieva, S. and Bieberich, E. (2011) The Gut-to-Breast Connection—Interdependence of Sterols and Sphingolipids in Multidrug Resistance and Breast Cancer Therapy. *Anti-Cancer Agents in Medicinal Chemistry*, **11**, 882-890. http://dx.doi.org/10.2174/187152011797655168

[25] Ogretmen, B. and Hannun, Y.A. (2004) Biologically Active Sphingolipids in Cancer Pathogenesis and Treatment. *Nature Reviews Cancer*, **4**, 604-616. http://dx.doi.org/10.1038/nrc1411

[26] Bieberich, E. (2004) Integration of Glycosphingolipid Metabolism and Cell-Fate Decisions in Cancer and Stem Cells: Review and Hypothesis. *Glycoconjugate Journal*, **21**, 315-327. http://dx.doi.org/10.1023/B:GLYC.0000046274.35732.47

[27] Apostolou, P., Toloudi, M., Chatziioannou, M., Ioannou, E. and Papasotiriou, I. (2012) Cancer Stem Cells Stemness Transcription Factors Expression Correlates with Breast Cancer Disease Stage. *Current Stem Cell Research & Therapy*, **7**, 415-419. http://dx.doi.org/10.2174/157488812804484639

[28] Kim, M.Y., Oskarsson, T., Acharyya, S., Nguyen, D.X., Zhang, X.H., Norton, L. and Massague, J. (2009) Tumor Self-Seeding by Circulating Cancer Cells. *Cell*, **139**, 1315-1326. http://dx.doi.org/10.1016/j.cell.2009.11.025

[29] Cristofanilli, M., Broglio, K.R., Guarneri, V., Jackson, S., Fritsche, H.A., Islam, R., Dawood, S., Reuben, J.M., Kau, S.W., Lara, J.M., Krishnamurthy, S., Ueno, N.T., Hortobagyi, G.N. and Valero, V. (2007) Circulating Tumor Cells in Metastatic Breast Cancer: Biologic Staging beyond Tumor Burden. *Clinical Breast Cancer*, **7**, 471-479. http://dx.doi.org/10.3816/CBC.2007.n.004

[30] Garrigan, E., Belkin, N.S., Alexander, J.J., Han, Z., Seydel, F., Carter, J., Atkinson, M., Wasserfall, C., Clare-Salzler, M.J., Amick, M.A. and Litherland, S.A. (2013) Persistent STAT5 Phosphorylation and Epigenetic Dysregulation of GM-CSF and PGS2/COX2 Expression in Type 1 Diabetic Human Monocytes. *PLoS ONE*, **8**, e76919. http://dx.doi.org/10.1371/journal.pone.0076919

[31] Nelson, J.D., Denisenko, O. and Bomsztyk, K. (2006) Protocol for the Fast Chromatin Immunoprecipitation (ChIP) Method. *Nature Protocols*, **1**, 180-185. http://dx.doi.org/10.1038/nprot.2006.27

[32] Inman, S. (2013) Breakthrough Designation Granted to Entinostat for Advanced Breast Cancer. OncLive. www.onclive.com

[33] Hait, N.C., Allegood, J., Maceyka, M., Strub, G.M., Harikumar, K.B., Singh, S.K., Luo, C., Marmorstein, R., Kordula, T., Milstien, S. and Spiegel, S. (2009) Regulation of Histone Acetylation in the Nucleus by Sphingosine-1-Phosphate. *Science*, **325**, 1254-1257. http://dx.doi.org/10.1126/science.1176709

[34] Yardley, D.A., Noguchi, S., Pritchard, K.I., Burris III, H.A., Baselga, J., Gnant, M., Hortobagyi, G.N., Campone, M., Pistilli, B., Piccart, M., Melichar, B., Petrakova, K., Arena, F.P., Erdkamp, F., Harb, W.A., Feng, W., Cahana, A., Taran, T., Lebwohl, D. and Rugo, H.S. (2013) Everolimus plus Exemestane in Postmenopausal Patients with HR[+] Breast Cancer: BOLERO-2 Final Progression-Free Survival Analysis. *Advances in Therapy*, **30**, 870-884. http://dx.doi.org/10.1007/s12325-013-0060-1

[35] Chia, S., Gradishar, W., Mauriac, L., Bines, J., Amant, F., Federico, M., Fein, L., Romieu, G., Buzdar, A., Robertson, J.F., Brufsky, A., Possinger, K., Rennie, P., Sapunar, F., Lowe, E. and Piccart, M. (2008) Double-Blind, Randomized Placebo Controlled Trial of Fulvestrant Compared with Exemestane after Prior Nonsteroidal Aromatase Inhibitor Therapy in Post-Menopausal Women with Hormone Receptor-Positive Advanced Breast Cancer: Results for EFECT. *Journal of Clinical Oncology*, **26**, 1664-1670. http://dx.doi.org/10.1200/JCO.2007.13.5822

[36] NCI (2013) FDA Approval for Vorinostat. Cancer Drug Information. Cancer Topics. www.cancer.gov

[37] Celgene Biopharmaceutical Press Release (2011) FDA Grants Accelerated Approval of ISTODAX® As Treatment for Patients with Peripheral T-Cell Lymphoma Who Have Received at Least One Prior Therapy. Celgene. Ir.celgene.com. Published Online. 16 June 2011.

[38] Campbell, R.M. and Tummino, P.J. (2014) Cancer Epigenetics Drug Discovery and Development: The Challenge of Hitting the Mark. *Journal of Clinical Investigation*, **124**, 64-69. http://dx.doi.org/10.1172/JCI71605