Targeting Thyroid Hormone Mediated Cancer Stem Cell Expansion, Treatment Resistance in ER+ Breast Cancer Patients

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ABSTRACT

Background: Breast cancer (BC) and thyroid disease are well recognized comorbidities, although pathogenesis of this relationship is not well understood. Hyperthyroidism reportedly promotes BC, whereas hypothyroid patients have shown a reduction in BC incidence. We have reported interactions between thyroid hormone replacement therapy (THRT) and BC in two historical cohorts (n=802 and n=160) of early stage, node negative BC and have shown THRT is significantly and independently associated with shorter disease-free and overall survival only in patients with steroid receptor positive (SR+) disease (Wahdan-Alaswad RS *et. al.*, 2020). Patients who received tamoxifen (Tam) and THRT had the worst survival. Our preclinical studies support that estrogen and thyroid hormones cross-talk by increasing cell proliferation, but the mechanism remains elusive. We aimed to study the pro-oncogenic signaling between TH and estrogen and understand how TH may enhance cell proliferation, stem-expansion, modulate hormone-associated gene regulation, and alters responsivity to endocrine therapy in ER+ BC.

Design: Two clinical cohorts of LN- SR+ BC patients were used to determine the effect of THRT on overall survival using Kaplan-Meier methods. We tested bi-directional cross-talk between TH and E2 using different BC cell lines, ER+ PDX *in vivo* models, *in vitro* methods, and publically available *in silico* data for modeling.

Results: Our results show that TH increase cell proliferation, enhances cell cycle and hormone-associated oncogenic signaling in SR+/ER+ BC when combined with estrogen. E2+TH enhanced nuclear co-localization and activation of ER-target genes such as GREB1 in ER+ BC cells. Knockdown of ESR1 or THRA decreased E2+TH-induced cell proliferation in ER+ BC cells. In E2 stimulated ER+ PDX tumors, TH significantly enhanced tumor growth and this was not attenuated when combined with tamoxifen (Tam) (P<0.0001 vs E2+Tam alone). RNAseq analysis these tumors show a marked increase in cell cycle regulatory genes (E2F1, Cyclin A, Cyclin E, and MYC) and when combined with tamoxifen a marked increase pro-oncogenic pathways (Wnt/Fizzled, MMPs, and TCL/LEFT) was observed. Thyroid hormone expanded stem cell expression of CD44, BMI-1, TGF-beta, and alternate stem genes. While TH is shown to enhance E2-mediated cell growth, use of tamoxifen did not dampen tumor growth whereas a full ER-antagonist (Fulvestrant) attenuated E2-TH mediated cross-talk.

Conclusions: These findings suggest that TH may enhance oncogenic signaling and is associated with a significantly increase mortality risk in ER+/SR+ BC tumors. Exogenous TH adversely affects SR+ BC and not SR- BC. Understanding the mechanism of cross-talk between TH and E2 allows us to define novel therapeutic strategies that will facilitate rapid clinical application for ER+ BC patients currently taking THRT.

Reference: Exogenous Thyroid Hormone is Associated with Shortened Survival and Upregulation of High Risk Gene Expression Profiles in Steroid Receptor Positive Breast Cancers. Reema S Wahdan-Alaswad, Susan M Edgerton, Hiba Salem, Hyunmin Kim, Aki-Choon Tan, Jessica Finlay-Schultz, Elizabeth Wellberg, Carol A. Sartorius, Britta M. Jacobsen, Bryan R Haugen, Bolin Liu, and Ann D Thor. DOI: 10.1158/1078-0432.CCR-20-2647.

RESULTS

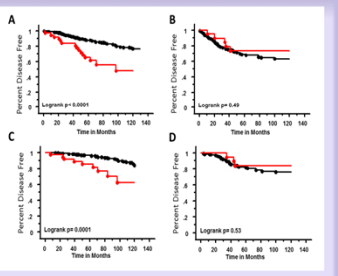

Figure 1. Thyroid hormone decreases disease free and disease specific overall survival in SR+ BC. (**A**) DFS for all SR+ patient by thyroid hormone treatment. Black circles represent no thyroid treatment (THRT), n= 533 patients, 84 relapsed, 84% relapse free (DF); red circles represent THRT patients, n = 29, 14 relapsed, 52% DF. (**B**) DFS for SR- patients by thyroid treatment. Black circles represent no THRT, n= 169 patients, 53 relapsed, 69% DF; red circles represent THRT, n = 18, 5 relapsed, 72% DF. (**C**) Disease specific survival (DSS) for all SR+ patients by thyroid treatment. Black circles represent no THRT, n = 530 patients, 45 dead of disease (DOD), 92% alive at last follow-up; red circles represent THRT, n = 29, 9 DOD, 69% alive at last follow-up. (**D**) DSS for all SR- patients by thyroid treatment. Black circles represent no THRT, n = 166 patients, 32 DOD, 81% alive at last follow-up; red circles represent THRT, n = 18 patients, 3 DOD, 83% alive at last follow-up.

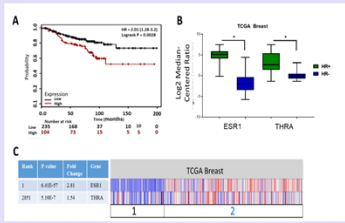

Figure 2. Expression of THRA is a poor prognostic indicator in ER+ BC. (**A**) DFS for SR+ patients by expression of THRA (204180_s0) in a total of 339 patients [1]. Red line signifies high expression of THRA (n=104) and black line represents low THRA (n=235) expression, P=0.0028. (**B**) Microarray data analysis of ESR1 and THRA expression in human breast cancer. ESR1 and THRA mRNA levels in hormone receptor HR+ (green) or HR- (green) breast cancer tissue from TCGA data set in Oncomine. * P<0.0001 by Tukey's t-test. (**C**) Relative expression of ESR1 and THRA in estrogen receptor (ER) negative (1) and ER positive (2) BC samples using TCGA Breast.

Factor	# patient	# events	Chi square	Δchi square	p value
DFS					
Base = age + size	568	107	22.293		
Base + TH	568	107	35.255	12.962	0.0003
DSS					
Base = size + grade	493	58	15.502		
Base + TH	493	58	22.988	7.486	0.0062
DFS 10 yr					
Base = age + size	568	97	22.522		
Base + TH	568	97	36.828	14.306	0.0002
DSS 10 yr					
Base = size	565	53	8.42		
Base + TH	565	53	17.5	9.08	0.0026
DFS 5 yr					
Base = size	568	61	7.886		
Base + TH	568	61	19.285	11.399	0.0007
DSS 5 yr					
Base = size	565	22	8.473		
Base + TH	565	22	12.837	4.364	0.0367

Table 2. Thyroid hormone is an Independent Predictor of Survival SR+ Stage I BC patients.

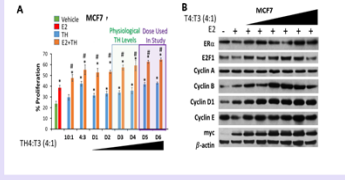

Figure 3. TH enhances ER+ BC cell proliferation. (**A**) MCF7 (ER+) cells were treated with vehicle (ETOH), E2 ($1x10^{-8}$M), T4 ($1x10^{-8}$ M to $1x10^{-10}$ M) and T3 ($2.5 x10^{-8}$ M to $2.5 x10^{-11}$ M) in a dose-dependent manner or with TH (10:1 ratio, T4 $1x10^{-8}$ M T3 $1x10^{-9}$ M) or TH (4:1 ratio T4 $1x10^{-8}$ M T3 $2.5x10^{-9}$M) with or without E2 ($1x10^{-8}$M). Cells were monitored for cell proliferation using live kinetic Incucyte Zoom® assay. Physiological levels outlined in green box and doses used in the study (purple) outlined. *P<0.001 relative to vehicle control, #P<0.001 relative to E2 alone. (**B**) ER+ BC MCF7cells were treated with vehicle (ETOH) or E2 ($1x10^{-8}$M) alone or in combination with T4 ($1x10^{-8}$M to $1x10^{-10}$M) and T3 ($2.5 x10^{-8}$ M to $2.5 x10^{-11}$ M) at 4:1 ratio. Cells were collected and harvested for Western blot analysis of cell cycle regulated protein expression relative to β-actin loading control.

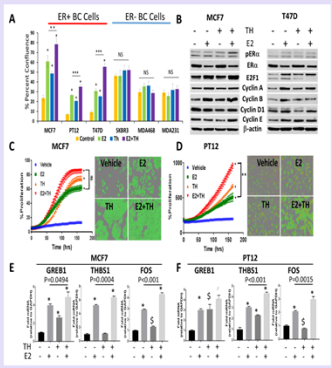

Figure 4. TH enhances ER+ BC cell proliferation, activation of cell cycle regulatory proteins a activates nuclear receptor target genes. (**A**) ER+ BC (MCF7, PT12, T47D) or ER- BC (SKBR3, MDA-MB-468, MDA-MB-231) cells were treated with vehicle (ETOH), E2 ($1x10^{-8}$M), TH (T4: $1x10^{-7}$ M, T3: $2.5x10^{-8}$ M at a 4:1 ratio) or combination of E2+TH and monitored for percent cell proliferation for 5 days by Incucyte Zoom® assay. (**B**) MCF7 (left) and T47D (right) were treated with vehicle (ETOH), E2 ($1x10^{-8}$M), TH (T4: $1x10^{-7}$ M, T3: $2.5x10^{-8}$ M at a 4:1 ratio) or combination of E2+TH, collected and harvested for Western blot analysis of cell cycle related proteins relative to β-actin loading control. ER+ BC MCF7 (**C**) and PT12 (**D**) cells were treated with vehicle (ETOH), E2 ($1x10^{-8}$M), TH (T4: $1x10^{-7}$ M, T3: $2.5x10^{-8}$ M at a 4:1 ratio) or combination of E2+TH and monitored for changes in cell proliferation over time using Incucyte Zoom® assay (left). #P<0.0001 relative to E2 alone. Live images at 140 hours were taken using Incucyte Zoom® 10x objective for percent confluence (right). MCF7 (**E**) and PT12 (**F**) cells were treated as described in E&F, collected and harvested for mRNA and assayed by qRT-PCR for relative change in mRNA expression of GREB1 THBS1, and FOS target genes. All experiments are done in triplicates, * P<0.0001 relative to control, *** P<0.001, # P<0.01, **P<0.01, *** or $ P<0.01.

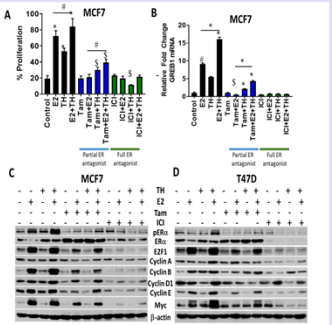

Figure 5. Thyroid hormone enhances endocrine therapy resistance to tamoxifen but not fulvestrant in ER+ BC cells. ER+ BC cell line MCF7 (**A**) treated with vehicle (ETOH), E2 ($1x10^{-8}$M), TH (T4: $1x10^{-7}$ M, T3: $2.5x10^{-8}$ M at a 4:1 ratio) or combination of E2+TH with or without tamoxifen (Tam, 1 mM) or fulvestrant (ICI 182,780, 1 mM) for 5 days and monitored over time for changes in proliferation as monitored by Incucyte Zoom®. (**B**) MCF7 cells were treated as outlined in Figure 3C for 18 hrs and harvested mRNA to examine relative fold change in GREB1 gene expression by qRT-PCR. MCF7 (**C**) or T47D (**D**) ER+ BC cells were treated as outlined in Figure 3C for 24 hrs prior to harvesting cell lysate for Western blot assay to examine relative fold change in protein expression of phospho-ERα, ERα or cell cycle regulatory proteins relative to β-actin loading control.

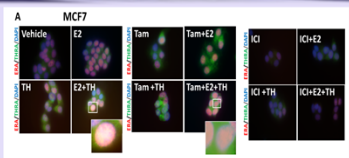

Figure 6. Estrogen and thyroid hormones enhances co-localized expression of THRA and ESR1 in the nucleus in ER+ BC cells. MCF7 (**A**) and PT12 (**B**) ER+ BC cells were treated as defined in Figure 3C for 24 hr prior to fixing cells and immunofluorescent staining of ERα (red), THRα (green) and DAPI (blue). Images of treated cells were taken on Nikon microscope at 100x magnification. Scale bar = 25 uM.

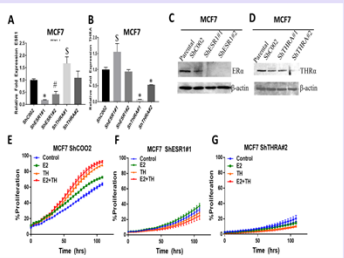

Figure 7. Knockdown of THRA and ESR1 in ER+ BC cells inhibits cell growth. Relative fold expression of ESR1 was evaluated in cells infected with either lentiviral control (ShCOO2), ESR1 (SHESR1#1 or SHESR1#2) or THRA (SHTHRA#1 or SHTHRA#1) and examined by qRT-PCR in MCF7 (**A**). Relative fold expression THRA was determined in aforementioned constructs and examined by qRT-PCR in MCF7 (**B**). Data are representative biological replicates. Relative fold expression of ERα or THRα protein expression relative to β-actin were assayed by WB (**C&D**). Changes in proliferation over time were observed in MCF7 cells transfected with aforementioned lentiviral constructs in the presence or absence of E2, TH or combination of E2+TH (**E-G**).

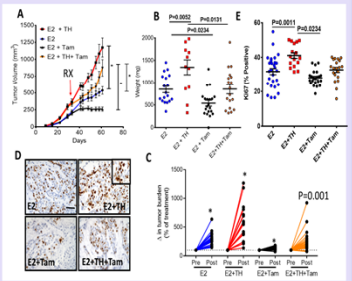

Figure 8. Thyroid hormone enhances E2-driven tumor growth and increases tamoxifen resistance in ER+ PDX tumor model. (**A**) Growth curves of mammary tumors after tumor implantation of ER+ PDX (UCD12-PDX) and treatment E2 along with TH, Tam or Tam +TH. Data represented as mean ± SEM (n=10 per group). (**B**) Weight of each of the tumors upon sacrifice at 60 days. (**C**) Tumor burden per mouse prior to (Pre) and after (Post) treatment (E2, E2+TH, E2+Tam, E2+TH+Tam). Data is calculated as percent change from treatment volume *P<0.0001. (**D**) Representative images of Ki67 staining for proliferation from UCD12-PDX tumors for each treatment group, Inset shows Ki67 positive staining. Magnification 40x. Bar is representative of 100 µm. (**E**) Ki67 quantification with Aperio Digital Pathology System of UCD12-PDX tumors is plotted as mean ± SEM.

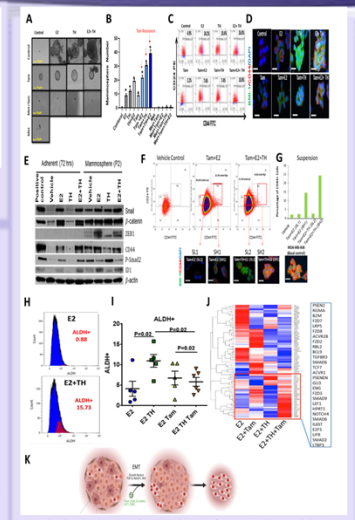

Figure 9. Thyroid hormone enhances stem cell expansion and expression of stem genes. Second generation (2P) mammospheres were generated using MCF7 cells treated as described Fig 3. Mammospheres were enumerated (**A**) by treatment group. (**B**) 2P mammospheres were collected, stained for stem markers (CD24-PE or CD44-FITC), and processed by flow cytometry. Luminal population (red) and basaloid/stem-like (pink) are highlighted and indicated by percentage of basaloid cells in upper right quadrant. (**C & D**) MCF7 cells were treated as described in Fig. 3 were fixed, stained for stem-markers CD44, red; BMI-1, green; and DAPI, blue by IFC, and examined using Nikon fluorescent microscope at 100x. Bar represents 30 µM. Experiment are triplicates ± SE. *P: <0.001, # P: <0.02 relative to E2+TH. (**E**) MCF7 Adherent cells or 2P mammospheres stimulated with vehicle, E2, TH, or E2+TH (conc. Described in Fig. 3) collected and stained for EMT proteins and analyzed by WB. (**F &G**) 2P mammospheres treated with vehicle, Tam+E2 or Tam+E+TH (concentrations similar to Fig.3) collected and flow sorted for sorted low (SL) or sorted high (SH) subpopulations and examined for expression of CD44 (right bar graph) or CSC markers BMI1 and CD44 (lower images). Experiments performed in triplicate. (**H**) E vs TH tumor stained with ALDH vs DAEB for ALDH+ cells. (**I**) ALDH+ tumors expression in different treatment responses. (**J**) Heatmap of stem cell gene expression in E2 vs ET+TH tumor samples. (**K**) Schematic diagram of TH-treated tumor enrichment for stem/mesenchymal genes.

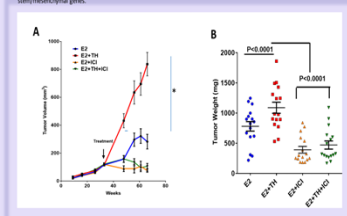

Figure 10. Direct attenuation of estrogen signaling using fulvestrant inhibits thyroid-mediated tumor growth in ER+ PT12 tumor model. (**A**) Growth curves of mammary tumors ER+ PT12 cells treated E2 along with TH, ICI or ICI+TH. Data represented as mean ± SEM (n=10 per group). (**B**) Wight (mg) of each of the tumors upon sacrifice at 66 days. Data represented as mean ± SEM.

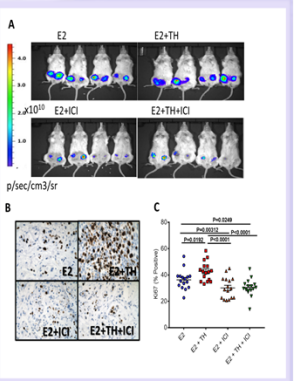

Figure 11. Direct attenuation of estrogen signaling using fulvestrant inhibits thyroid-mediated tumor growth in ER+ PT12 tumor model. (**A**) Tumor volume was monitored using IVIS after injection of D-luciferin at 60 days. Representative mice from each cohort is shown. (**B**) Representative images of Ki67 staining for proliferation from PT12 tumors for each treatment group, Inset shows Ki67 positive staining. Magnification 40x. Bar is representative of 100 µm. (**C**) Ki67 quantification with Aperio Digital Pathology System of PT12 tumors is plotted as mean ± SEM.

CONCLUSIONS

1. THRT is independently associated with a significantly shortened disease free and disease specific overall survival in early stage, chemo-naive SR+ BC patients.
2. Thyroid Hormone Receptor A (THRA) gene expression is higher in SR+ as compared to SR- BC cases. Amongst SR+ patients, high expression of THRA is associated with a shorter DFS. is a poor prognostic indicator in this subset of all BCs.
3. TH alone or in combination with E2 enhances cell proliferation, activation of cell cycle regulatory genes and proteins only in ER+ BC cells *in vitro and in vivo*.
4. In ER+ BC cells TH reduces Tam mediated growth inhibition, whereas ICI activity is less effected by concomitant TH.
5. Knockdown of THRA and ESR1 attenuates TH-induced ER+ BC cell proliferation, indicating a major role for these receptors in cross-talk signaling.
6. TH enhances E2 driven tumor growth in an ER+ PDX BC model, and interferes with Tam associated tumor growth inhibition, validating findings from our observations patient data.
7. TH enhances expression of pro-tumorigenic pathways and activation of cell cycle regulatory genes.
8. Complete attenuation of estrogen signaling by ICI blocks TH-mediated tumor growth.

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