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Targeting Thyroid Hormone Receptor Beta in Triple Negative Breast Cancer

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Abstract

Purpose—Discover novel nuclear receptor targets in triple negative breast cancer

Methods—Expression microarray, western blot, qRT-PCR, MTT growth assay, soft agar anchorage-independent growth assay, TRE reporter transactivation assay, statistical analysis.

Results—We performed microarray analysis using 227 triple negative breast tumors, and clustered the tumors into five groups according to their nuclear receptor expression. Thyroid hormone receptor beta (TR β) was one of the most differentially expressed nuclear receptors in group 5 compared to other groups. TR β low expressing patients were associated with poor outcome. We evaluated the role of TR β in triple negative breast cancer cell lines representing group 5 tumors. Knockdown of TR β increased soft agar colony and reduced sensitivity to docetaxel and doxorubicin treatment. Docetaxel or doxorubicin long-term cultured cell lines also expressed decreased TR β protein. Microarray analysis revealed cAMP/PKA signaling was the only KEGG pathways upregulated in TR β knockdown cells. Inhibitors of cAMP or PKA, in combination with doxorubicin further enhanced cell apoptosis and restored sensitivity to chemotherapy. TR β -specific agonists enhanced TR β expression, and further sensitized cells to both docetaxel and doxorubicin. Sensitization was mediated by increased apoptosis with elevated cleaved PARP and caspase 3.

Conclusions—TR β represents a novel nuclear receptor target in triple negative breast cancer; low TR β levels were associated with enhanced resistance to both docetaxel and doxorubicin treatment. TR β -specific agonists enhance chemosensitivity to these two agents. Mechanistically enhanced cAMP/PKA signaling was associated with TR β 's effects on response to chemotherapy.

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Conflict of Interest

Authors have no conflicts of interest to disclose.

Keywords

Thyroid hormone Receptor β ; chemotherapy; cyclic AMP; Protein Kinase A; triple negative breast cancer

Introduction

Progress has been made in treating patients with estrogen receptor alpha (ER α) or HER2-positive breast tumors [1–3]. However, triple negative breast cancer (TNBC) (ER α -/progesterone receptor (PR)-/HER2-) is harder to treat as these tumors exhibit high proliferation rates, early recurrence, and decreased disease survival [4,5]. TNBC is currently treated with chemotherapy due to the lack of ER and HER2 therapeutic targets [6–8]. Many TNBCs are sensitive to chemotherapy and treatment improves disease-free and overall survival [8,9]. However, the problem of chemotherapy resistance is still a significant clinical problem [10,11].

Taxanes, doxorubicin, and platinum compounds are used in TNBC [12–15]. Unfortunately there are few useful predictors of chemotherapeutic response [16,17]. Since chemotherapy is often limited by toxicity, there is a need to explore biologic targets in this subtype. A recent genomic study identified gene pathways associated with chemotherapy sensitivity in breast cancer subtypes [18], and in the ER-negative subgroup, gene sets involved in elevated G-protein signaling, fatty acid synthesis, and cAMP-mediated signaling were associated with poor chemotherapeutic response [18]. Some TNBCs express elevated levels of the androgen receptor (AR), and one TN cell line has been shown to be androgen-responsive [19]. A molecular luminal AR (LAR) subgroup has also been defined in TNBC, and corresponding cell lines are responsive to AR antagonist treatment [20]. In a Phase II TNBC trial with AR-expressing tumors, AR targeted therapy demonstrated a 19% clinical benefit rate [21]. Thus, with the success of targeting AR in TNBC, we hypothesized that other nuclear receptors (NRs) could be novel therapeutic targets in TNBC.

There are 48 members of the NR superfamily, and a recent study reported that many NR levels were lower in tumors compared to normal breast tissues [22]. It has been shown that thyroid hormone receptor beta (TR β) acts as a tumor suppressor of invasion and metastasis in TNBC cell lines [23]. TR β is a nuclear transcription factor that mediates the pleiotropic activities of thyroid hormones, T3 and T4, and influences basal oxygen consumption, cardiac contractility, and lipid metabolism [24–26]. Additionally, activation of TRs with T3 modulates aerobic glycolysis (the Warburg effect) in breast cancer cells [27]. TR β levels, but not the closely related TR α isoform, were negatively associated with tumor grade [22]. TR β promotes transcription in the presence of thyroid hormones, but inhibits transcription in absence of ligand [25]. Importantly specific TR β agonists are available, and have been clinically successful in lowering triglyceride and cholesterol levels without inducing side effects in the heart which are mediated by TR α [28–30].

We recently reported RNA expression profiling of 198 TNBC and defined TNBC subtypes with distinct prognostic outcomes [31]. We used these array data to subtype TNBC using NR expression levels, and found that the TRs were overexpressed in one group, and elevated

TR β levels were associated with better clinical outcomes. The role of TR β in breast cancer is not well studied. We discovered that low TR β levels were associated with resistance to selected chemotherapeutic drugs, and treatment with a specific TR β agonist restored chemo sensitivity in TNBC cells.

Results

Low levels of TR β are associated with poor clinical outcomes and enhanced growth of TNBC

Treatment of TNBC suffers from the lack of clinical targets or known genetic drivers limiting gene-targeted approaches [32]. In order to explore whether NRs could be potential targets in TNBC, we used supervised hierarchical clustering to separate 227 TNBC into 5 potential subgroups (Supplemental Fig. 1A). NRs contributing to these clusters were scored using Prediction Analysis of Microarray (PAM) across the clustered groups, and the average expression of the NRs determined (Fig. 1A). Group 5 represents 24% of the total patients, and the most highly-expressed receptors classifying this group are shown in Fig. 1B. Previous analysis of this microarray dataset using non-negative matrix factorization [31] classified AR-overexpressing tumors as the luminal AR (LAR) group defined by Lehman [20]. Luminal AR tumors are known to have abundant AR protein, are ER protein negative, but ER RNA positive. However, our NR-classified group 5 tumors do not overlap with Lehmann's LAR group signature [20] (data not shown), but correspond to a group with high expression of several NRs, including AR, ER, COUP TF1, TR β , and ERRs α/γ (Fig. 1B). To determine which TNBC cell line models could be used to explore the role of TR β , we matched our available cells to the NR subgroups 1–5 using PAM classification [33], and examined levels of group 5 NRs in the cell lines using qRT-PCR and immunoblot analysis (Fig. 1C and D). Corresponding NR group 5 cell lines MDA-MB-453, HCC2185 and HCC202 showed higher levels of TR β protein compared to NR group 3. Levels of AR were also higher in NR group 5 tumors, with no ER protein expressed as expected.

We first evaluated correlations between TR β expression and patient survival using publicly available clinical data [34]. TNBC patients with high TR β mRNA levels were associated with longer disease-free survival (Fig. 1E). Since the majority of ER-negative patients in this dataset were treated with different chemotherapeutic regimens, we cannot differentiate TR β effects on prognosis independent of treatment.

TR β can affect invasion and metastasis in MDA-MB-486 ER-negative breast cancer cells [23]. To explore the role of TR β on the growth of TNBC cells, we used shRNA knock down (KD) in three representative cell lines, and soft agar or MTT assays performed. HCC202 and MDA-MB-453 KD cells formed significantly more colonies compared with cells infected with empty vector (Fig. 1F, EV). MTT assays in HCC2185 confirmed these results (Fig. 1G). Inducible overexpression of TR β in MDA-MB-453 cells reduced cell growth as expected and enhanced chemosensitivity (Supplemental Fig. 2). Our data suggest that TR β could act as a tumor suppressor in TNBC cells.

TR β KD enhances resistance to chemotherapy and blocks apoptosis of TNBC cells

To evaluate the effect of TR β levels on response to chemotherapy regimens commonly used in TNBC, we determined IC₅₀ values in TR β KD and EV cells after treatment for 3 days with the taxane docetaxel (T), the anthracycline doxorubicin (D), or cisplatin (C). TR β KD increased the IC₅₀ values for docetaxel and doxorubicin in both cell lines (Fig. 2A and B, respectively). IC₅₀ values for docetaxel and doxorubicin were increased 5–6 fold and 4–7-fold, respectively, in HCC2185 and HCC202 cells. However, the IC₅₀ for cisplatin did not change with TR β KD. To test whether drug treatment might also affect TR β expression, we cultured NR group 5 cell lines for 2 months in drug. TR β protein levels decreased in all three cell lines with continuous docetaxel and doxorubicin treatments (Fig. 2C–E). In HCC202 and MDA-MB-453 cells, TR β levels decreased in a dose-dependent manner. These data suggest that treatment itself can decrease TR β levels, which could further contribute to resistance and low TR β expression could be a predictive marker for reduced response to chemotherapy.

To explore mechanisms underlying resistance induced by TR β KD, we performed immunoblot analysis to focus on apoptosis induced by chemotherapy. Cysteine-dependent aspartate-directed proteases (caspases) are a family of polypeptides that cleave key cellular proteins to affect the apoptotic cascade [35]. Activated caspase-3 can then cleave poly (ADP-ribose) polymerase (PARP) and block DNA repair [36]. TR β KD cells dramatically reduced PARP and caspase 3 cleavages with docetaxel and doxorubicin (Fig. 2F and G). Reduction in PARP and caspase-3 cleavage was observed with cisplatin treatment, although no change was seen in IC₅₀ for this drug. These data suggest that low TR β -expressing tumors may be more resistant to chemotherapy via a reduction in cellular apoptosis.

Cyclic AMP-PKA signaling is involved in TR β -mediated resistance

We used microarray expression profiling to determine which cellular pathways were altered with TR β KD in HCC2185 cells. We found 266 probes were differentially expressed between KD and EV (Supplemental Table 1; FDR < 0.1; n=3 per group). We used DAVID [37] to identify pathways that were enriched in KD cells (148 probes shown in bold). Only the chemokine signaling pathway, including adenylate cyclase 2 which catalyzes the formation of cyclic adenosine monophosphate (cAMP), was significantly enriched (Fig. 3A, p=0.038, Supplemental Fig. 3 and Table 1). In a pooled analysis of breast cancer patients treated with a number of different chemotherapy regimens, it has been shown that cAMP-mediated signaling, along with several other pathways such as fatty acid and xenobiotic metabolism, were the most consistently associated with resistance to chemotherapy (14), leading us to further examine cAMP signaling in our TR β KD model.

Since cAMP is involved in activation of protein kinase A (PKA), we studied their roles in TR β -mediated resistance. We used a cAMP synthesis inhibitor (Fig. 3B and C), and the competitive PKA inhibitor H89 (Fig. 3D and E), in combination with chemotherapy, in cell growth assays. Both inhibitors were able to significantly decrease growth in combination with doxorubicin in EV HCC2185 and HCC202 cells. The inhibitors were also able to restore sensitivity to doxorubicin in both TR β KD cells. We did not observe inhibitors affecting docetaxel response (data not shown). As expected TR β KD increased PKA

phosphorylation in both lines (Fig. 3F with the densitometry for PKA, PARP, caspase 3 lanes shown in panel G and Supplemental Fig. 4). H89 in combination with doxorubicin significantly decreased both total and phosphorylated PKA (pPKA), which was most noticeable in the HCC202 TR β KD cells. Chemotherapeutic drugs inhibit cancer cell growth partly through activation of apoptosis [38]. We therefore performed western blot analysis to evaluate apoptosis in chemotherapy treated cells using cleaved caspase-3 and PARP antibodies (Fig. 3F and G). Doxorubicin alone increased cleaved caspase-3, and the cleavage was further increased with doxorubicin in combination with H89 in both HCC202 (Fig. 3F) and HCC2185 cells (Supplemental Fig. 4). These data suggest that inhibition of cAMP/PKA signaling blocks TR β -mediated resistance. This class of inhibitors might be useful to enhance sensitivity to chemotherapeutics, such as doxorubicin, or perhaps to enable the use of lower, less toxic doses.

TR β specific agonists enhance sensitivity to chemotherapy through apoptotic mechanisms

T3 is the endogenous TR β ligand, but it can also bind to TR α and induce serious side effects to both the heart and muscles. Therefore we tested the effects of two selective TR β agonists, GC-1 and KB-141, on cell growth [30,29]. We first determined whether these TR β agonists could activate canonical thyroid hormone response elements (TRE) in HCC202 EV and KD cells (Supplemental Fig. 5A and B). GC-1 and KB141 both activated TRE activity in HCC202 EV cells, but not KD cells. Steroid receptor ligands can often modulate receptor levels, and we found that both agonists enhanced TR β protein levels (Fig 4A and Supplemental Fig. 6A). These data demonstrate that GC-1 and KB-141 may stabilize TR β receptor protein in these cell lines.

Since higher TR β levels were associated with chemosensitivity (Fig. 2A and B), and TR β specific agonists enhanced TR β expression (Fig. 4A), we next evaluated the role of GC-1 and KB-141 on docetaxel and doxorubicin sensitivity. GC-1 or KB-141 did not affect basal cell growth, but docetaxel treatment decreased growth by 50% (Fig. 4B). Docetaxel, in combination with GC-1 or KB-141, significantly decreased cell viability (from 50% to 15%). GC-1 treatment also decreased proliferation in combination with doxorubicin (Fig. 4C). These data suggest that TR β -specific agonists enhance chemosensitivity, and therefore lower doses of chemotherapy could be used in patients. These agonists might thus be useful to reduce the known toxicity of chemotherapy.

We next evaluated the effect of TR β agonists on apoptosis using western blot analyses. Cells were treated with KB-141, chemotherapy or in combination. Since KB-141 was the most effective at enhancing TR β expression and chemosensitivity, we tested it in combination with docetaxel or doxorubicin (Fig. 4D/E and F, respectively). Docetaxel treatment alone enhanced PARP cleavage which was further increased when combined with KB-141. We observed enhanced cleaved caspase 3 when docetaxel was combined with KB-141 (Fig 4E). KB-141 similarly increased cleaved PARP and cleaved caspase 3 in combination with doxorubicin (Fig. 4F). These cumulative data suggest that enhanced apoptosis underlies the growth inhibitory effects of combined treatment which enhances chemosensitivity.

Discussion

There are few biomarkers which predict chemotherapeutic response in TNBC. NRs are attractive targets because there are agonists and antagonists available, and several TN subtypes overexpress specific NRs such as TR β . TR β is a potent nuclear transcription factor which modulates a significant number of genes expressed in brain, liver, and kidney tissues [39]. Herein we hypothesized that TR β levels could predict sensitivity to chemotherapy. We discovered that low TR β levels were indeed associated with poor clinical outcomes and enhanced growth of TNBC, and specific knockdown of TR β enhanced resistance to both docetaxel and doxorubicin treatment via blockade of apoptosis. In addition, treatment with these agents alone also modulated TR β levels, thus treatment may initiate this resistance mechanism. Furthermore, we found that TR β -mediated resistance involved cyclic AMP and PKA signaling, which could be blocked with specific agents. These data suggest that patients with low TR β expression might be relatively more resistant to specific therapeutic regimens, and might benefit from TR β -targeted therapies in combination with chemotherapy.

Tumors with high levels of TR β were associated with elevated levels of other NRs, such as AR and ESR1, but this group does not correspond to the luminal AR TN subtype defined by Lehmann et al [20]. High TR β levels were associated with better outcomes in breast cancer, and we thus hypothesized that elevated levels might influence therapeutic response. Indeed, when we reduced TR β levels we saw significant effects on chemotherapeutic sensitivity with changes in IC50 values for both docetaxel and doxorubicin. A recent study demonstrated that thyroid hormone levels were lower in chemotherapy-treated breast cancer patients, and in preclinical breast cancer models, treatment with T3 increased drug sensitivity [40]. However, the use of T3 in patients to augment therapeutic response is contraindicated because of toxic effects on the heart. A thyroid hormone analog, eprotirome (KB2115), has been tested in a clinical trial to lower LDL in patients with hypercholesterolemia, and did not demonstrate negative side effects on the heart [41]. We have not tested this agent, but selected two TR β -specific agonists GC-1 and KB-141 to determine their effects on response. Treatment with these agonists significantly enhanced sensitivity to docetaxel and doxorubicin, and increased cellular. Our data suggest that TR β -specific agonists in combination with chemotherapy may be useful without initiating deleterious effects of TR α stimulation.

In order to explore downstream mechanisms underlying TR β 's effects on chemosensitivity, we evaluated gene expression changes after knockdown of TR β . Chemokine signaling was the only KEGG pathway significantly increased in TR β KD cells, and cAMP/PKA gene signaling was increased in this pathway by TR β KD. We confirmed this result demonstrating that TR β KD cells expressed higher levels of phosphorylated PKA. cAMP activation inhibited doxorubicin-induced apoptosis in leukemia cells through effects on p53 [42]. In ER-negative patients, cAMP-mediated signaling was one of the top pathways associated with chemotherapy response [18]. To test the role of cAMP/PKA activation in our models, we employed a cAMP inhibitor, bupivacaine, and the PKA inhibitor H89 alone and in combination with doxorubicin. Both of these inhibitors restored sensitivity to doxorubicin in all cells. Our data suggest that activation of cAMP/PKA signaling might underlie TR β 's role

in response to chemotherapy. It is tempting to speculate that PKA inhibitors might be useful to enhance chemotherapeutic response in low TR β -expressing TN tumors.

In summary, low TR β levels may predict response to specific chemotherapy regimens that are commonly used in TNBC. In addition, TR β -specific agonists might be used in combination with docetaxel or doxorubicin to enhance sensitivity and to lower doses and decrease toxicity and improve quality of life measures during treatment.

Material and methods

Reagents and antibodies

Thiazolyl Blue Tetrazolium Bromide (MTT), 3, 3', 5 Triiodothyronine and puromycin were obtained from Sigma (St. Louis, MO). MEM, RPMI 1640, DMEM, L-glutamine, penicillin/streptomycin, SeaPlaque™ Agarose was from Lonza (Walkersville, MD). Fetal bovine serum was from Gemini Bio Products, (West Sacramento, CA). Lipofectamine LTX, SuperScript® III Reverse Transcriptase and qPCR probes (ESR1, THRB, COUP-TF1 and GAPDH) were provided by Life Technologies (Grand Island, NY). The renilla luciferase assay kit was from Promega (Madison, WI). TR β and GAPDH antibodies were from Santa Cruz Biotechnology (Dallas, TX). Total ER α antibody was from Vector Laboratories (Burlingame, CA). AR, c-PARP, PARP, c-Caspase 3, Caspase 3, pPKA and PKA antibodies were from Cell Signaling Technology (Beverly, MA). ChemiGlow Chemiluminescent Substrate kit was from Protein Simple (Santa Clara, CA). Docetaxel and doxorubicin were from LC Laboratories (Woburn, MA). Cisplatin was from ENZO (Plymouth Meeting, PA). c-AMP inhibitor bupivacaine and H89 were obtained from Selleck Chemicals (Houston, TX)..

Plasmids

pGIPZ-shTR β and pGIPZ-EV plasmids were from Thermo Scientific (Pittsburgh, PA). LV113 empty vector and TR β inducible plasmids were from Genecopoeia (Rockville, MD). TRE renilla reporter was from Switchgear Genomics (Carlsbad, CA). The pLightSwitch-LR plasmid contained synthetic TREs (direct repeat) separated by four nucleotides *TGACCCcagctgAGGTCAAGGTCAcaggAGGTCAAGGTCAATGACCT*. The pLVX-Tet3G plasmid was from Clontech Laboratories, Inc (Mountain View, CA).

Cells and stable transfection

HCC202, HCC38, HCC1569, HCC2185 and ZR75-B were cultured in RPMI 1640 containing 10% fetal bovine serum. MDA-MB-453 and BT474AZ cells were grown in DMEM containing 10% fetal bovine serum. MCF-7 cells were cultured in MEM containing 10% fetal bovine serum. Cell medium contained 0.1 mM nonessential amino acids, and 100 I.U./mL penicillin and 100 μ g/mL streptomycin. The cells were maintained at 37°C in the presence of 5% CO₂.

HCC202, HCC2185 and MDA-MB-453 cells were plated in 75 cm² flasks for one day and then transfected with 10 μ g of pGIPZ plasmids containing TR β shRNA or shEV in regular growth medium. After 48 hour transfection, t medium was replaced with 1 μ g/ml puromycin

for selection. Stable pools were collected for further analysis. The MDA-MB-453 TR β inducible cell line was created using two steps: parental cells were transduced with pLVX-Tet3G and pools selected with neomycin to stably express Tet protein. pLVX-Tet3G stable pools were transduced with lentivirus vector (LV-113) containing the TRE promoter conjugated with TR β cDNA and selected with puromycin.

Cell extraction and immunoblot analysis

HCC202, MDA-MB-453, HCC2185, HCC38, HCC1569 and MCF-7 cells were lysed in 100 μ l of RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 0.1% SDS, 1 mM sodium orthovanadate plus 1:100 proteinase inhibitor cocktail III (Calbiochem, La Jolla, CA). Protein concentrations were determined using BCA Protein Assay kits (Pierce, Rockford, IL) according to manufacturer's directions. Equal amounts of total protein were resolved under denaturing conditions on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with primary antibodies. For cleaved caspase 3 expression, an 11% gel was used instead. To ensure equal loading, all membranes were incubated with anti-GADPH or RhoGDI α antibodies. Secondary antibodies were applied to the membranes for 1 hour at room temperature and developed with enhanced chemiluminescence reagents (Protein Simple, San Jose, CA).

Anchorage-independent growth assays

Cells (2×10^3 /well) were plated in 2 ml of 0.35% agarose in phenol red-free media, on top of 0.7% of agarose base layer in 24-well plates. One day after plating, media containing vehicle or treatments as indicated were added to the top layer, and replaced every two days. After 14 days colonies $>50\mu$ m in diameter were counted with GelCount. Each condition was performed at least in triplicate.

MTT growth assays

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, Sigma) assays. Cells (2×10^3 cells/well) were plated in 96-well plates and treated as indicated. 50 μ l of MTT stock solution (5 mg/ml in PBS) was added to each well and incubated at 37°C for 2 hours. The media was removed and 100 μ l of dimethyl sulphoxide (DMSO) was added to each well. After shaking the plates for 20 minutes, the absorbance at 570 nM was measured with background subtraction at 655 nM. Each condition of treatment was performed at least in triplicate.

TRE transactivation assays

HCC202 or HCC2185 cells were plated in 24-well plates for one day and transfected with TRE Renilla Reporter and β -Gal. 24 hours after transfection, cells were treated for an additional 24 hours with T3, GC-1 or KB141 in charcoal-stripped medium. β -galactosidase plasmid was used to normalize the efficiency of the transfection. Renilla luciferase reporter activities were measured by the Renilla Luciferase kit following manufacturer's protocol. Transfections were performed in triplicate.

Quantitative Reverse Transcription-PCR (qRT-PCR) assays

Gene expression was evaluated by quantitative reverse transcription PCR (RT-PCR) method. Cells were grown in 6-well plates to 70%–80% confluency and total RNA extracted using RNeasy kits (Qiagen). 250 ng of total RNA was reverse transcribed in a final volume of 20 μ l using Superscript III first-strand kits (Invitrogen), and cDNA was amplified by PCR using the following primers: TR β , AR, ER α and GAPDH. Each condition was performed in triplicate.

Microarray analysis

RNA was extracted using RNeasy micro kits (Qiagen). Labeled cRNA was hybridized onto Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix Inc. Santa Clara, CA) in triplicate. Chips were normalized with probeset level estimates of expression previously generated using tools in Bioconductor (www.bioconductor.org) and/or BRB Array tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>)

Statistical analyses

Data were analyzed for statistical significance ($P < 0.05$) using two-tailed Student's Test using GraphPad Prism 5. Standard deviations (SD) are shown. Multivariate Cox proportional hazards model and Kaplan–Meier curve were used for patients DFS analysis in Sabatier *et al.* dataset [34].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR	Androgen receptor
CIS or C	Cisplatin
DOC or T	Docetaxel
DOX or D	Doxorubicin
ERα	Estrogen receptor alpha
EV	empty vector
KD	Knockdown
LAR	luminal AR
NRs	Nuclear receptors

PAM	Prediction analysis of microarrays
TRβ	Thyroid hormone receptor beta
TNBC	Triple negative breast cancer

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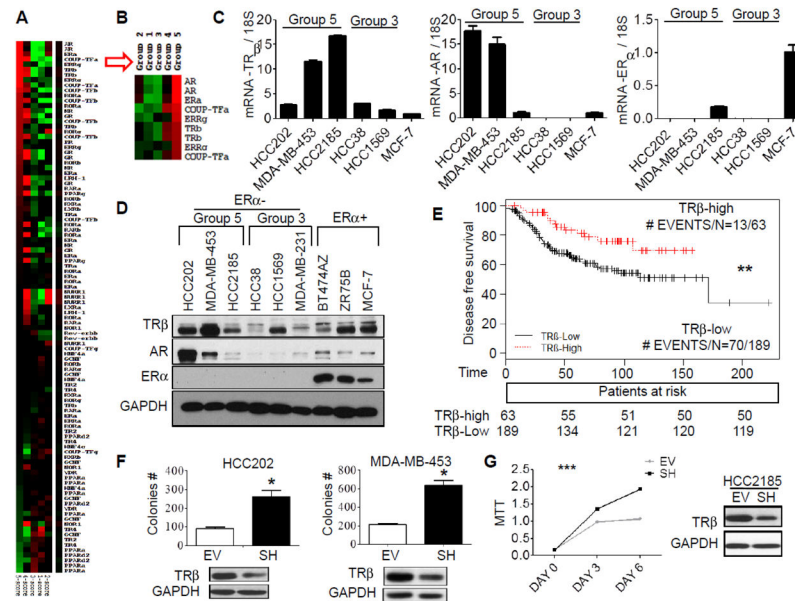


Figure 1. Low TR β levels are associated with worse DFS and enhanced cell proliferation

A. Heatmap showing comparison of NR gene expression among five patient groups using Cluster 3.0 and TreeView. The comparison was calculated based on relative NR gene expression. Red or green color indicates relatively high or low expression respectively, compared to other groups, and black indicates average expression. Some genes were recognized by multiple Affymetric probes. **B.** The most differentially-overexpressed genes in group 5 are shown. **C.** qRT-PCR was performed to evaluate the gene expression of TR β , AR and ER α in group 5 representative cell lines HCC202, MDA-MB-453, and HCC2185. 18S RNA was used as a loading control. Group 3 representative cell lines HCC38 and HCC1569 were used as negative controls. MCF-7 gene amplification cycles were used as the reference to calculate fold change. **D.** TR β , AR, and ER α protein expression was examined using western blot analysis in group 5, group 3, and ER positive breast cancer cell lines. GAPDH was used as loading control. **E.** Kaplan-Meier plot of TR β using Sabatier *et al.* [34] (** $p < 0.01$). Data represent an average of 3 Affymetric TR β probesets, and 75th percentile was used as the cut-point to separate patients into two outcome groups. **F.** HCC202 and MDA-MB-453 cells were stable transfected with empty vector (EV) or TR β shRNA (SH) plasmid and TR β expression was evaluated by western blot, and soft agar. Results represent the average \pm SD of three experiments normalized to respective EV (* $p < 0.05$). **G.** HCC2185 EV and SH cells were analyzed for TR β expression using western blot analysis and growth in MTT assays using GraphPad Prism 5 (** $P < 0.001$ SH cells growth curve compared to EV).

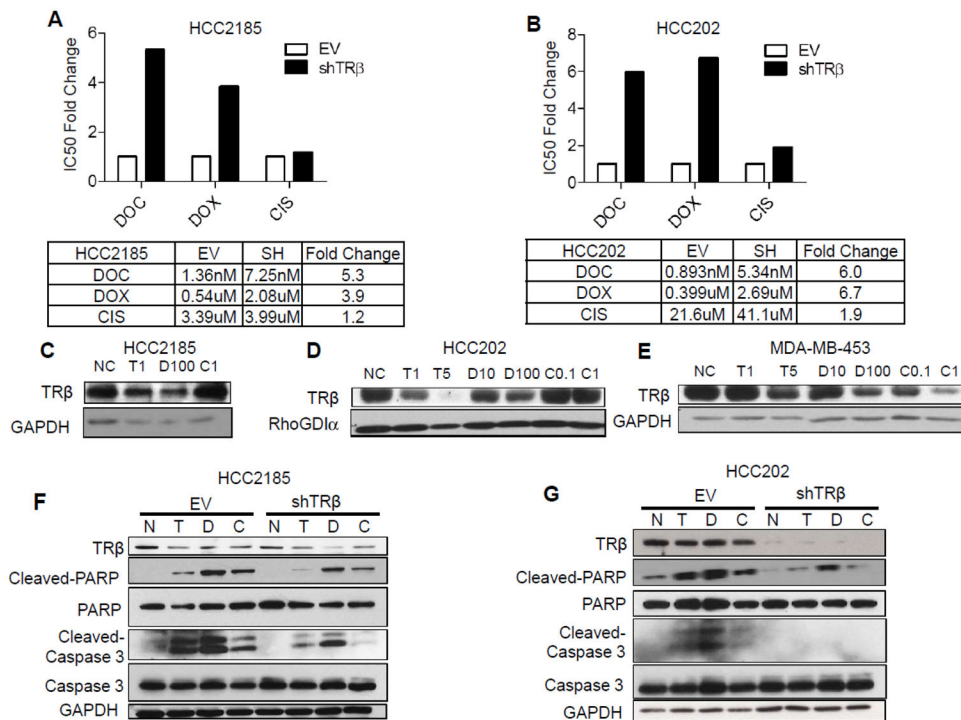


Figure 2. Knockdown of TR β enhances resistance to docetaxel and doxorubicin

A, B. HCC2185 and HCC202 cells (EV and shTR β) were treated with DOC, DOX or CIS for 3 days and IC₅₀ values were calculated using MTT growth assays performed in triplicate. **C, D, E.** HCC2185, HCC202 and MDA-MB-453 cells were treated with DOC 1 or 5 nM (T1 and T5, respectively), DOX 10 nM or 100 nM (D10 or D100, respectively) or CIS 0.1 uM or 1 uM (C0.1 or C1, respectively) for 2 months, and endogenous TR β protein levels were measured using western blot. GAPDH or RhoGDI α levels were used as loading controls. **F, G.** HCC2185 and HCC202 cells (EV and shTR β) were treated with DOC 1 nM (T), DOX 100 nM (D) and CIS 1 uM (C) for 4 days, and then western blots were performed for TR β , cleaved PARP, PARP, cleaved caspase 3 and caspase 3 expression. GAPDH was used as a loading control.

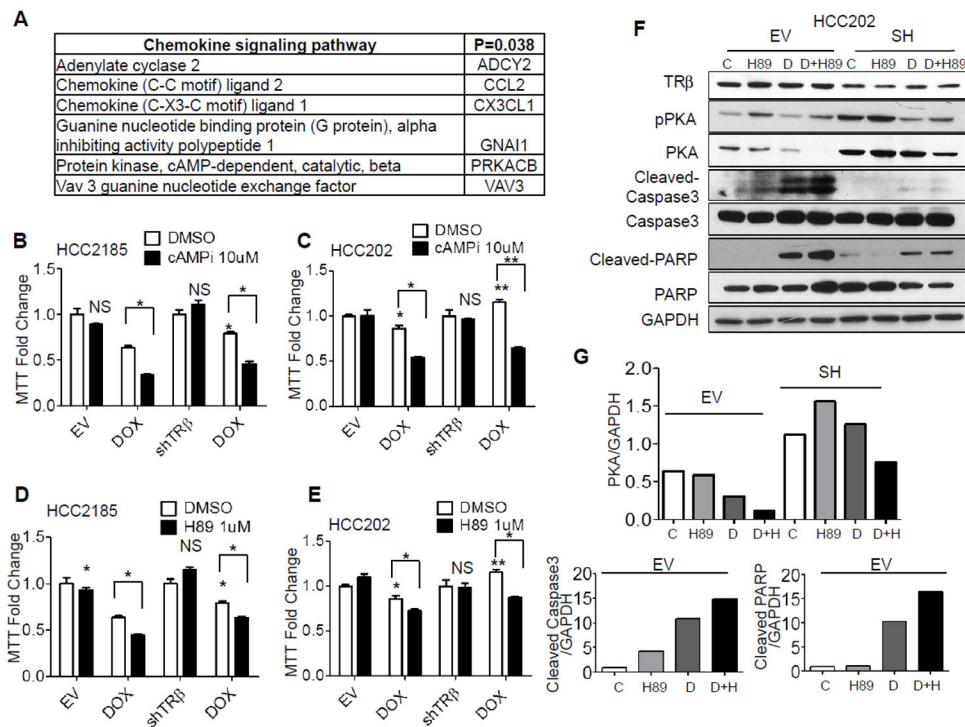


Figure 3. TR β -mediated resistance involves cAMP/PKA signaling

A. Genes from the top KEGG pathway, chemokine signaling, in TR β KD cells compared to EV group were identified using DAVID. **B and C.** HCC2185 and HCC202 cells (EV and shTR β) were treated with DOX, a cAMP inhibitor, alone or in combination for 3 days, and MTT growth assays were performed.* p<0.05 and ** p<0.01. **D and E.** HCC2185 and HCC202 cells were treated with DOX or the PKA inhibitor H89, alone or in combination and growth assessed using MTT assays. **F.** HCC202 cells were treated with H89 (1 uM) or Dox (200 nM), alone or in combination for 4 days, and analyzed by western blot analysis. TR β , pPKA, PKA, cleaved caspase 3, caspase 3, cleaved PARP and PARP expression were examined using GAPDH as a loading control. **G.** Densitometry of western blot shown in panel F.

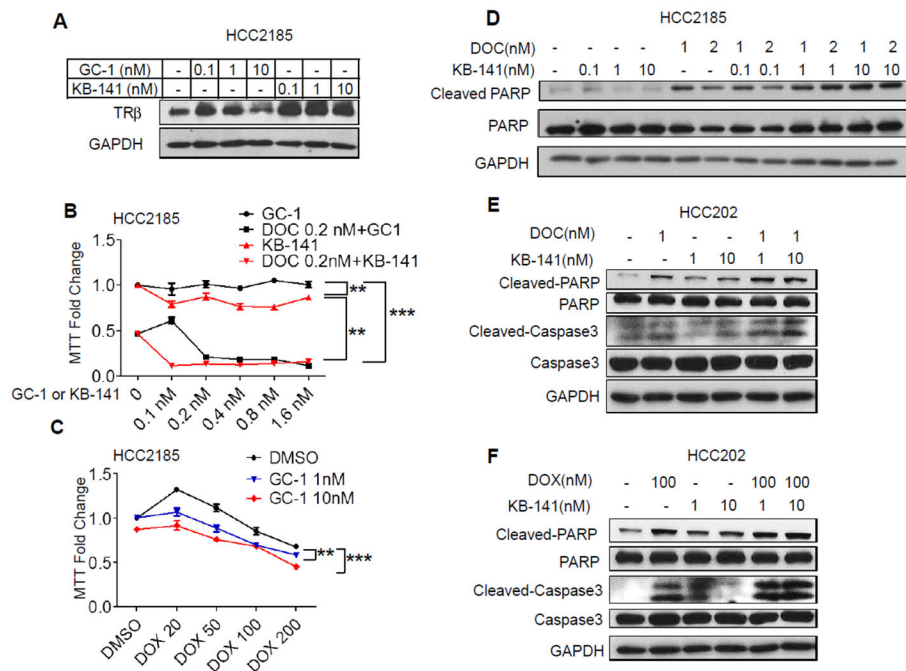


Figure 4. TR β agonists enhance sensitivity to DOC and DOX

A. HCC2185 cells were treated with GC-1, KB-141 or vehicle for 5 days and western blot analysis performed. GAPDH was used as the loading control. **B.** HCC2185 cells were treated with DOC (0.2 nM) alone, or in combination with GC-1 or KB-141 (0.1, 0.2, 0.4, 0.8 or 1.6 nM) for 9 days, and then growth was assessed using MTT assays. Results are expressed as fold change \pm SD relative to vehicle treated cells (** $p < 0.01$ and *** $p < 0.001$). **C.** HCC2185 cells were treated with DOX (20 nM, 50 nM, 100 nM or 200 nM) or GC-1 (1 nM or 10 nM) or in combination for 6 days, and MTT growth assay was performed to measure cell viability (** $p < 0.01$ and *** $p < 0.001$). **D.** HCC2185 cells were treated with Doc (1 or 2 nM) in combination with KB-141 (0.1 nM, 1 nM or 10 nM) for 6 days. Western blot analysis was performed for cleaved PARP and total PARP; GAPDH was used as a loading control. **E.** HCC202 cells were treated with DOC, KB141 alone or in combination for 6 days, and analyzed for cleaved PARP, total PARP, cleaved caspase 3, and total caspase 3 levels; GAPDH was used as loading control. **F.** HCC202 cells were treated with DOX or KB-141 alone or in combination. Western blot analysis was performed to measure cleaved PARP, PARP, cleaved caspase 3 and caspase 3. GAPDH was used as loading control.