

Overexpressing modified human TR β 1 suppresses the proliferation of breast cancer MDA-MB-468 cells

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Abstract. A number of studies have indicated that thyroid hormone receptor β 1 (TR β 1) functions as a tumor suppressor. TRs mediate transcriptional responses through a highly conserved DNA-binding domain (DBD). A novel rat TR β isoform (rTR $\beta\Delta$) was previously identified, in which a novel exon, N (108 bp), is located between exons 3 and 4 within the DBD; this exon represents the only difference between rTR $\beta\Delta$ and rTR β 1. *In vitro*, rTR $\beta\Delta$ exhibits a stronger tumor-suppressive capacity than rTR β 1, and further analysis revealed a high level of conservation between the rat and human DBD sequences. In the present study, an artificially modified human TR β 1 (m-hTR β 1) was constructed via the introduction of the 108-bp sequence into the corresponding position of the wild-type human TR β 1 (wt-hTR β 1) DBD. An electrophoretic mobility shift assay and transfection experiments confirmed that m-hTR β 1 is functional. Overexpression of m-hTR β 1 inhibits the proliferation of MDA-MB-468 cells in the presence of triiodothyronine by promoting apoptosis, which may be associated with the upregulation of *Caspase-3* and *Bak* gene expression and the activation of the Caspase-3 protein. In addition, the pro-apoptotic effect of m-hTR β 1 was stronger, compared with wt-hTR β 1. These results indicated that m-hTR β 1 may act as a tumor suppressor in MDA-MB-468 cells. These data provided a novel insight into gene therapy for breast cancer.

Introduction

Thyroid hormone receptors (TRs) are members of a superfamily of nuclear receptors and ligand-dependent transcription factors. TRs are encoded by two genes, TR α (*THRA*) and TR β

(*THRB*) (1), and four triiodothyronine (T3) and DNA-binding receptor isoforms, TR α 1, TR β 1, TR β 2 and TR β 3, that are only present in rats, are generated by alternative mRNA splicing (2). These isoforms are considered functional receptors and share a common structure with several regions from the N- to the C-terminus, including: A N-terminal activation domain (A and B regions); a conserved DNA-binding domain (DBD) (C region); a hinge domain (D region); and a ligand-binding domain (E and F regions) (3). TRs can bind to thyroid hormone response elements (TREs) as homodimers or heterodimers via the retinoid X receptor (4). In addition to the regulation of metabolism, growth and development, increasing evidence has indicated that TRs have an important role in cell proliferation and malignant transformation (5). Low or no expression of TRs and alterations in TR genes, particularly TR β , have been identified in a number of cancer types (6,7). Studies have also demonstrated that mutations in TR β are closely associated with a number of cancer types (8,9).

Cell-based studies and xenograft models have confirmed that TR β , particularly TR β 1, functions as a tumor suppressor to suppress tumor cell proliferation, migration and tumorigenesis (5,10). A novel rat TR β isoform have confirmed TR $\beta\Delta$ (GenBank number: DQ191165) was previously identified. rTR $\beta\Delta$ is homologous to rTR β 1 and only contains an additional 108 nucleotides or 36 amino acids (11). Structural analysis has indicated that these additional 36 amino acids are located in the DBD, which alters this highly conserved domain (11). Nonetheless, previous studies (12,13) have demonstrated that rTR $\beta\Delta$ is a functional TR with transcription factor characteristics that exhibit greater tumor-suppressive ability *in vitro*, compared with rTR β 1. In the present study, the additional 36 amino acids were introduced into the corresponding position of wild-type human TR β 1 (wt-hTR β 1), which was termed modified human TR β 1 (m-hTR β 1); then, the transcription factor characteristics and *in vitro* anti-tumor effects of m-hTR β 1 were examined in human breast cancer MDA-MB-468 cells without endogenous TR β .

Materials and methods

Cells and reagents. MDA-MB-468 and MCF-7 human breast cancer cells were obtained from the Cell Bank of the Chinese

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Academy of Sciences (Shanghai, China). Chitosan (CS), with a molecular mass of 60,000-100,000 Da (degree of deacetylation ~85%) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). L-15 cell culture medium, an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, NE-PER™ Nuclear and Cytoplasmic Extraction Reagents and an anti-TR β -1 antibody (cat. no. MA1-216; dilution, 1:1,000) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A Caspase-3 spectrophotometric assay kit (cat. no. G007) was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, Jiangsu, China). Antibodies against Histone H3 (cat. no. bs-0349R; dilution, 1:1,000), active Caspase-3 (cat. no. bsm-33199M; dilution, 1:200) and Bak (cat. no. bs-1284R; dilution, 1:200) were purchased from BIOCSS (Beijing, China). M-MLV reverse transcriptase, TRIzol® total RNA extraction reagent and quantitative real-time polymerase chain reaction (PCR) detection kits (SYBR® Premix Ex Taq™ II; cat. no. RR820B) were obtained from Takara Bio, Inc. (Otsu, Japan). KOD-Plus-Ver polymerase and the KOD-Plus-Mutagenesis kit were obtained from Toyobo Life Science (Osaka, Japan).

Construction of pcDNA3.1-wt-hTR β 1 and pcDNA3.1-m-hTR β 1 vectors. MCF-7 cells were cultured and collected. Total RNA was extracted using TRIzol reagent and was used for reverse transcription to generate cDNA. This 'total cDNA' was used as a template for PCR to obtain the full-length wt-hTR β 1 cDNA. The forward primer 5'-CCCAAGCTTATGACTCCCAACAGTATGAC-3' contains a restriction endonuclease site for *Hind*III (underlined), whilst the reverse primer 5'-CCGGAATTCCCTAATCCTCGAACACTTCC-3' contains an *Eco*RI site (underlined). The *Hind*III-*Eco*RI PCR product was subcloned into pcDNA3.1 (Invitrogen; Thermo Fisher Scientific, Inc.) at the corresponding sites. The constructed expression plasmid, pcDNA3.1-wt-hTR β 1, was confirmed by sequence analysis.

Mutant constructs were generated by site-directed mutagenesis. The following primers were used to generate mutations: Primer 1 5'-TCTACCTCTCTGCATTCGGTCTGGATGTTGTCCTCAAGGCAGTGGGACCCTAGGTTTCTTTAGAAGAACCATTCAGAAAAAT-3'; Primer 2 5'-ACTGGTGTCTGTATGGAACCAATCCCTGTCTTCTCGTCTCTGGTGTGAGAAGGCTTGACGCTTCACACGTGATACAGCGGT-3'. The underlined portion of the primers indicates the novel introduced 108-bp exon, and the non-underlined portion indicates the partial wt-hTR β 1 DBD sequence. Site-directed mutagenesis was performed using a KOD-Plus-Mutagenesis kit. This procedure involved PCR amplification using the pcDNA3.1-wt-hTR β 1 plasmid as the template and mutation primers were performed according to the manufacturer's instructions. The desired mutations were confirmed by DNA sequencing of the entire gene. The recombinant plasmid carrying the 108-bp exon was termed pcDNA3.1-m-hTR β 1.

Preparation of pDNA/nucleic kinase substrate short peptide (NNS) CS complexes. The nuclear localization signal-linked NNS was fused to CS to form the gene carrier^{NNS}CS as previously described (14). The empty plasmid pcDNA3.1 and the plasmids pcDNA3.1-wt-hTR β 1, pcDNA3.1-m-hTR β 1 and pGL3-TRE (the palindromic TRE is located upstream of the SV40 promoter in the pGL3-promoter vector, which contains a Luciferase

reporter gene) (11) were extracted, and the pDNA/^{NNS}CS complex (pcDNA3.1/^{NNS}CS, pcDNA3.1-wt-hTR β 1/^{NNS}CS, pcDNA3.1-m-hTR β 1/^{NNS}CS and pGL3-TRE/^{NNS}CS) was prepared as previously described (14).

Western blotting. MDA-MB-468 cells were seeded at a density of 1×10^6 cells/ml in the wells of a 6-well plate in L-15 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). When the cells had grown to 75% confluence, transfection were performed as below, and pcDNA3.1/^{NNS}CS, pcDNA3.1-wt-hTR β 1/^{NNS}CS and pcDNA3.1-m-hTR β 1/^{NNS}CS were added separately to the 6 wells to achieve DNA concentrations of 4 μ g/well. Cells were collected following 48 h of transfection, and nuclear proteins were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (cat. no. 78833; Thermo Fisher Scientific, Inc.). The nuclear protein levels were measured by BCA assay (cat. no. CW0014; CWBIO, Beijing, China) and each sample containing 30 μ g nuclear protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with TBS-T [25 mM Tris-Cl (pH 7.4), 150 mM NaCl and 0.05% Tween-20] containing 5% skimmed milk for 2 h at room temperature, and then incubated with primary antibodies against TR β 1 (cat. no. MA1-216; dilution, 1:1,000) and Histone H3 (cat. no. bs-0349R; dilution, 1:1,000). TR β 1 recognizes an epitope in the A/B domain of hTR β 1, namely, residues 1-101, which are common sequences of wt-hTR β 1 and m-hTR β 1. After being thoroughly washed in TBS-T, the membranes were incubated with the secondary antibodies, HRP-conjugated mouse anti-rabbit IgG (cat. no. bs-0295M-HRP; dilution, 1:5,000; BIOCSS) or Goat Anti-Mouse IgG, HRP Conjugated (cat. no. bs-0368G-HRP, dilution 1:5,000, BIOCSS) for 1.5 h at room temperature. After thorough washing with TBS-T, the membranes were visualized using an enhanced chemiluminescence kit (Merck KGaA) and Kodak films (Kodak, Rochester, NY, USA).

Electrophoretic mobility shift assay (EMSA). The DNA-binding abilities of wt-hTR β 1 and m-hTR β 1 were first determined using a synthetic DR4 TRE; the oligonucleotide sequences and labeling of the DR4 TRE probe were reported previously (11). Following the aforementioned transfection of MDA-MB-468 cells with pcDNA3.1-wt-hTR β 1/^{NNS}CS and pcDNA3.1-m-hTR β 1/^{NNS}CS for 72 h, nuclear proteins were extracted and subjected to standard EMSA analysis, as previously described (11).

Transcriptional activity analysis. pcDNA3.1/^{NNS}CS, pcDNA3.1-wt-hTR β 1/^{NNS}CS and pcDNA3.1-m-hTR β 1/^{NNS}CS were co-transfected with pGL3-TRE/^{NNS}CS to determine the transcriptional activity of wt-hTR β 1 and m-hTR β 1. A *Renilla* luciferase plasmid, pRL-TK (Promega Corporation, Madison, WI, USA), was included to correct for transfection efficiency. Following MDA-MB-468 cell transfection for 6 h, the transfection solution was discarded and replaced with serum-free medium, and 10 nM T3 (Sigma-Aldrich; Merck KGaA) was added to each T3 intervention group. Following 48 h of transfection (T3 exposure for 42 h), luciferase and

Renilla activities were assayed using a Dual-Glo[®] Luciferase Assay System (Promega Corporation) according to the manufacturer's instructions. Firefly luciferase activity was normalized to the *Renilla* controls. At the same time, each group of nuclear proteins was extracted using NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and the overexpression of wt-hTRβ1 and m-hTRβ1 protein was measured using a standard western blotting assay as aforementioned. The results demonstrated that the expression levels of wt-hTRβ1 and m-hTRβ1 for each condition are identical.

Cell proliferation and apoptosis assays. MDA-MB-468 cells were seeded at a density of 1×10^4 cells/ml in the wells of a 96-well plate. When the cells had grown to 75% confluence, pcDNA3.1/^{NS}CS, pcDNA3.1-wt-hTRβ1/^{NS}CS and pcDNA3.1-m-hTRβ1/^{NS}CS were added to the wells. Following 6 h of transfection, the transfection solution was discarded and replaced with serum-free medium IL-15, and 10 nM T3 was added to the intervention groups. At 48 h following transfection (T3 exposure for 42 h), MTT (Promega Corporation) solution (20 μl, 5 mg/ml) was added to each well, and the plate was incubated at 37°C for 4 h, following which the formazan crystals were solubilized in dimethyl sulfoxide (200 μl/well). The absorbance at 570 nm was recorded using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and background absorbance at 630 nm was subtracted.

MDA-MB-468 cells were seeded in wells of a 12-well plate and transfected as aforementioned. At 48 h following transfection (T3 exposure for 42 h), the cells were collected and stained with FITC-conjugated Annexin V and propidium iodide for 10 min at room temperature. The stained cells were then detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Bak, Bax and Caspase-3 expression assays. For the expression assay, MDA-MB-468 cells were transfected as aforementioned. At 48 h following transfection (T3 exposure for 42 h), the cells were collected and total RNA was extracted using TRIzol reagent. mRNA (2 μg) was reverse transcribed into total cDNA in a 20 μl reaction mixture using the PrimeScript[™] RT reagent kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Inc.). *Bak*, *Bax* and *Caspase-3* mRNA levels were analyzed by reverse transcription-quantitative PCR (RT-qPCR) and were detected with SYBR[®] Green using an iQ5[™] system (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 35 cycles at 95°C for 5 sec, 56°C for 20 sec and 72°C for 20 sec. Primers for each gene were as follows: *Bak*, forward 5'-TACCGCCATCAGCAGGAAC-3' and reverse 5'-TCTGAGTCATAGCGTTCGGTTG-3'; *Bax*, forward 5'-GGA GCTGCAGGATGATTG-3' and reverse 5'-GGCCTTGAG CCAAGTTTG-3'; *Caspase-3*, forward 5'-GGAAGCGAA TCAATGGACTC-3' and reverse 5'-TTCCCTGAGGTTTGC TGC-3'; and *Gapdh*, forward 5'-GCATCCTGGGCTACA CTGAG-3' and reverse 5'-CCACCACCCTGTTGCTGTAG-3'. The Cq values for all genes from different samples were collected. Raw data were normalized to *GAPDH* expression,

and the relative expression level of each gene is represented as $2^{-\Delta\Delta Cq}$ (15).

The expression levels of active Caspase-3 and Bak proteins were analyzed by western blot analysis as aforementioned. Antibodies against active Caspase-3 (cat. no. bs-0081R; dilution, 1:300), Bak (cat. no. bs-1284R; dilution, 1:300) and GAPDH (cat. no. sc-32233; dilution 1:1,000) (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used.

Caspase-3 activity assay. MDA-MB-468 cells were seeded in a 6-well plate and transfected as aforementioned. At 48 h after transfection (T3 exposure for 42 h), the cells were lysed using the Lysis Buffer in the Caspase-3 Spectrophotometric assay kits and the Caspase-3 activity was assessed using this kit according to the manufacturer's instructions.

Statistical analysis. For all measurements, the data are expressed as the mean ± standard deviation based on three independent experiments. The results were analyzed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for multi-group comparisons with the Student-Newman-Keuls as the post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

m-hTRβ1 construction, expression and functional analysis. The amino acid sequences of the human and rTRβ1 DBDs are highly conserved (16). rTRβΔ has an additional 108 bp sequence between exons 3 and 4, and the segment containing the additional 36 amino acids was located on the C-terminal side of the first two amino acids of the P-box (Fig. 1A) of the first zinc finger motif. This increased the length of the linker between the two zinc fingers by 3.1-fold (17 to 53 amino acids). The full-length coding sequence of wt-hTRβ1 (1368 bp) was successfully cloned into pcDNA3.1 to obtain the expression plasmid pcDNA3.1-wt-hTRβ1 (Fig. 1B). The coding sequence of the additional 36 residues (108 bp) was successfully introduced into a similar position in wt-hTRβ1 by site-directed mutagenesis, and this mutant was termed pcDNA3.1-m-hTRβ1 (Fig. 1B). The DBD of m-hTRβ1 is only 36 amino acids longer than wt-hTRβ1 (Fig. 1C).

To examine wt-hTRβ1 and m-hTRβ1 expression and activity, pcDNA3.1 (control), pcDNA3.1-wt-hTRβ1 and pcDNA3.1-m-hTRβ1 were transiently transfected into MDA-MB-468 cells. Protein expression was assessed by western blot. The two proteins (m-hTRβ1 and wt-hTRβ1) were ~57 and ~53 kDa, respectively, (Fig. 2A, lanes 2 and 3), and no TRβ was detected in the control transfection group (Fig. 2A, lane 1). The lower band depicted in Fig. 2 is Histone H3, which was used as a control. DNA binding was assessed by EMSA using a DR4 TRE. Nuclear proteins containing TRβ (wt-hTRβ1 and m-hTRβ1) can bind to DR4, as demonstrated by a shift in DNA electrophoretic mobility. These DNA binding assays were sequence-specific, as the receptors competed with a 100-fold excess of unlabeled DR4 (Fig. 2B). The EMSA results indicated that TRβ (wt-hTRβ1 and m-hTRβ1) receptors may bind to DR4.

The transcriptional activities of wt-hTRβ1 and m-hTRβ1 as T3 receptors were compared using a transient transfection

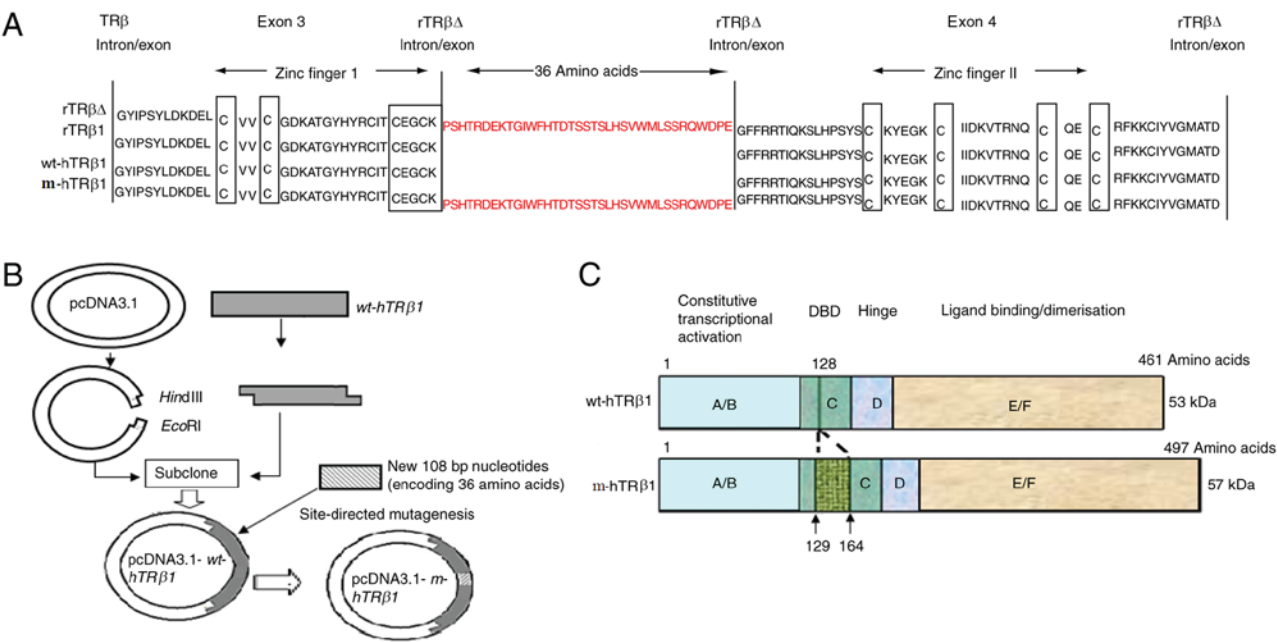


Figure 1. Schematic of the wt-hTRβ1 and m-hTRβ1 protein structure and expression vector construction. (A) Amino acid sequences around the P box in the DBD of TRβs. The amino acid sequences are presented from exon 3-4 of TRβ. Homologous sequences are relative to the zinc finger motifs. A long gap is introduced for best alignment. The P box and the cysteines of the zinc fingers are boxed. The coding sequence of the 36 amino acids was introduced between exon 3 and 4 of wt-hTRβ. The mutant was termed m-hTRβ1 (B) Map of the pcDNA3.1-wt-hTRβ1 and pcDNA3.1-m-hTRβ1 plasmids, vector construction and the mutation are depicted. (C) Schematic diagram representing the protein structure of wt-hTRβ1 and m-hTRβ1. TRβ, thyroid hormone receptor β; wt-hTRβ1, wild-type human TRβ1; m-hTRβ1, modified human TRβ1; rTRβΔ, rat TRβ isoform; DBD, DNA-binding domain.

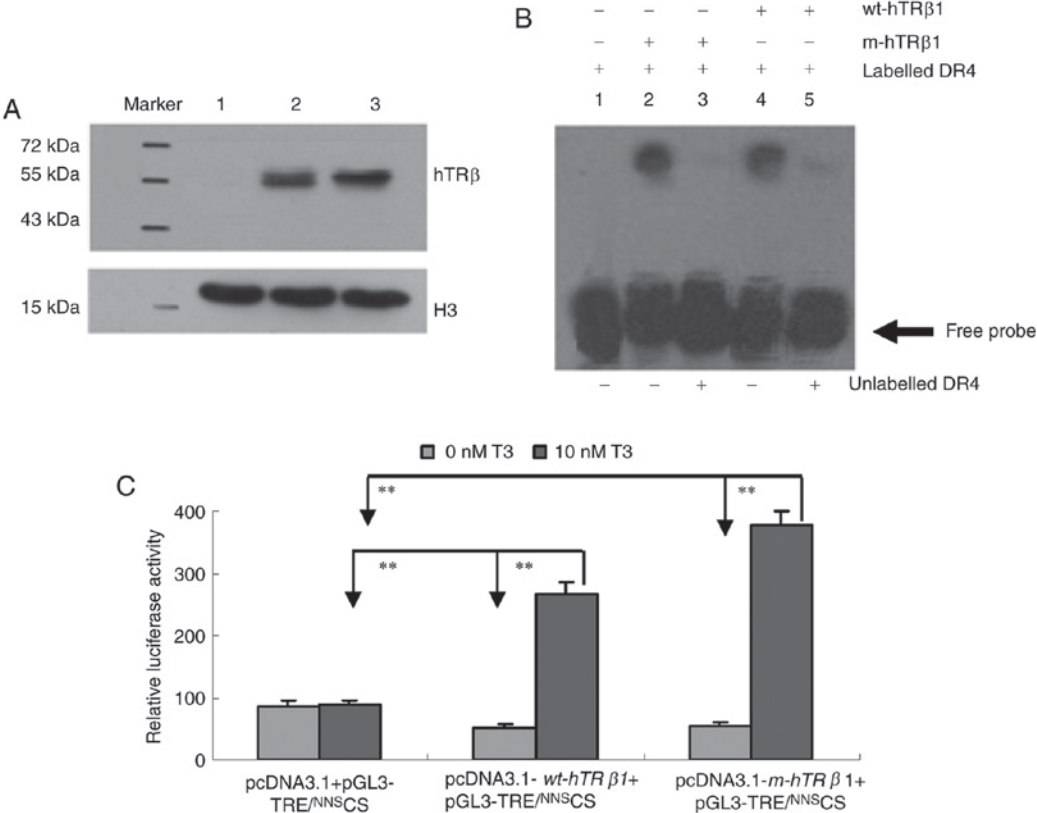


Figure 2. Expression and properties of the wt-hTRβ1 and m-hTRβ1 proteins. (A) Western blot detection of wt-hTRβ1 and m-hTRβ1 expression in MDA-MB-468 cells. Lane 1, pcDNA3.1/NNSCS; Lane 2, pcDNA3.1-m-hTRβ1/NNSCS; and Lane 3, pcDNA3.1-wt-hTRβ1/NNSCS. (B) DNA binding activity was measured by EMSA. Lane 1, digoxin-labeled DR4 (17 fmol/μl); lanes 2 and 4: digoxin-labeled DR4 (17 fmol/μl) incubated with m-hTRβ1 and wt-hTRβ1 in the absence of competitor (100-fold excess of unlabeled DR4); lanes 3 and 5: digoxin-labeled DR4 (17 fmol/μl) incubated with m-hTRβ1 and wt-hTRβ1 in the presence of competitor (100-fold excess of unlabeled DR4). (C) Luciferase activity in MDA-MB-468 cell lysates. Firefly luciferase activity was normalized to Renilla controls and data are presented as the mean ± standard deviation. **P<0.01. wt-hTRβ1, wild-type human TRβ1; m-hTRβ1, modified human TRβ1; TRE, thyroid hormone response elements; NNS, nucleic kinase substrate short peptide; CS, chitosan; T3, triiodothyronine.

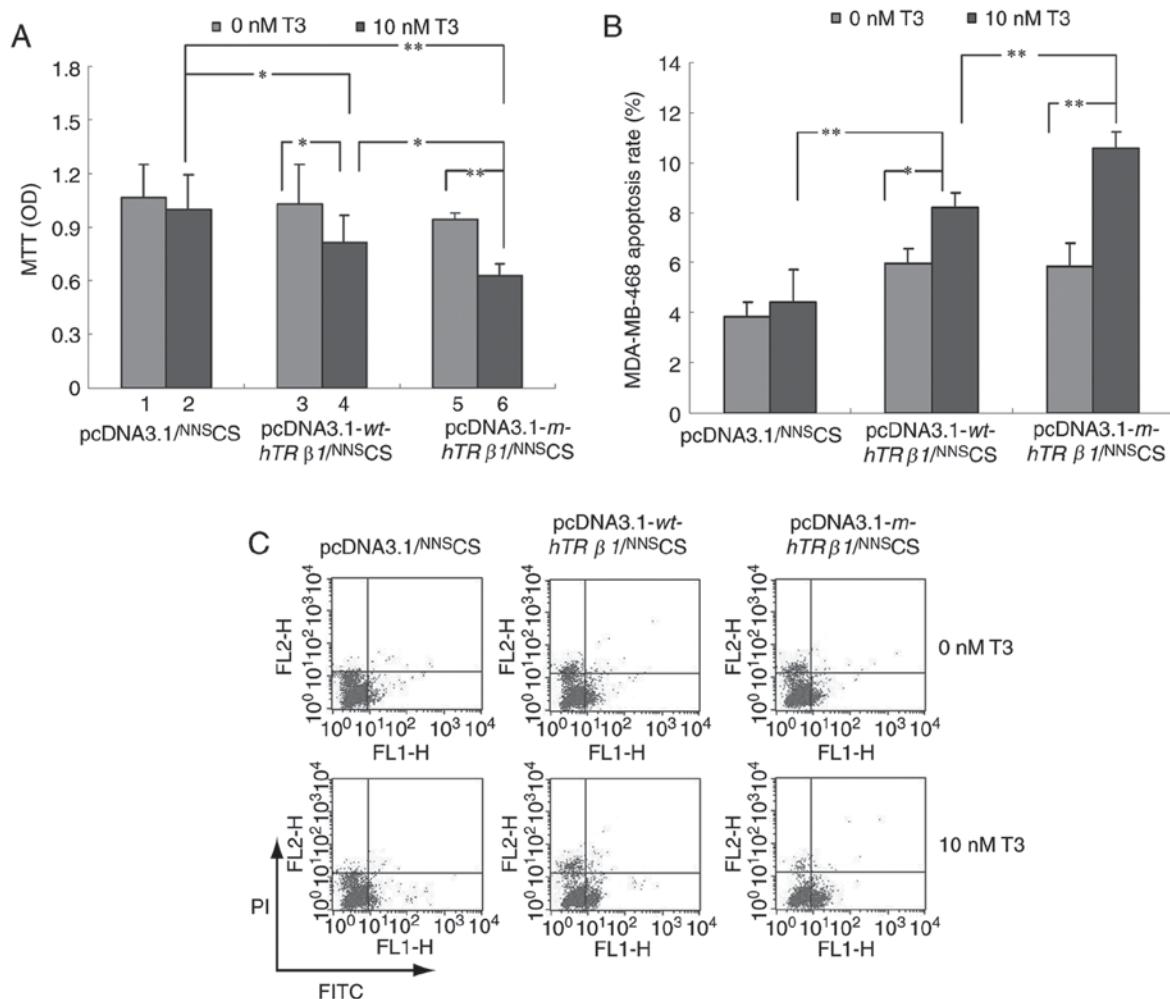


Figure 3. Effect of wt-hTRβ1 and m-hTRβ1 on the proliferation and apoptosis of MDA-MB-468 cells. (A) The proliferation of MDA-MB-468 cells following wt-hTRβ1 or m-hTRβ1-gene transfection was detected by MTT assay. Data are presented as the mean \pm SD. (B and C) The apoptosis of MDA-MB-468 cells following wt-hTRβ1 or m-hTRβ1-gene transfection was detected by Annexin V-FITC assay. The total number of apoptotic cells was determined by calculating the sum of early apoptotic cells (Annexin V-FITC+/PI-) and late apoptotic cells (Annexin V-FITC+/PI+) detected by flow cytometry (C); Analysis of the apoptosis rate of MDA-MB-468 cell (B); the data are presented as the mean \pm SD. * $P < 0.05$; ** $P < 0.01$. OD, optical density; wt-hTRβ1, wild-type human TRβ1; m-hTRβ1, modified human TRβ1; NNS, nucleic kinase substrate short peptide; CS, chitosan; T3, triiodothyronine; PI, propidium iodide; FITC, fluorescein isothiocyanate; SD, standard deviation.

system. Relative luciferase activity levels were assessed in each group. Transfection with pcDNA3.1+pGL3-TRE/NNSCS resulted in basic luciferase activity levels in the presence or absence of T3 ($P > 0.05$). The pcDNA3.1-wt-hTRβ1 + pGL3-TRE/NNSCS and pcDNA3.1-m-hTRβ1 + pGL3-TRE/NNSCS transfection group demonstrated a similar increase in luciferase activity levels in the absence of T3 ($P > 0.05$); these levels were increased 5.4- and 6.9-fold, respectively, following treatment with 10 nM T3 (Fig. 2C).

hTRβ1 inhibits MDA-MB-468 cell proliferation and promotes MDA-MB-468 cell apoptosis. To examine the effect of wt-hTRβ1 and m-hTRβ1 on cell proliferation, MDA-MB-468 cells were transiently transfected with pcDNA3.1/NNSCS, pcDNA3.1-wt-hTRβ1/NNSCS or pcDNA3.1-m-hTRβ1/NNSCS, and subjected to MTT assays. Fig. 3A depicted that T3 had no significant effect on MDA-MB-468 cell growth. However, the expression of wt-hTRβ1 and m-hTRβ1 inhibited cell proliferation in the presence of T3. Furthermore, the effect of m-hTRβ1 on cell proliferation inhibition was stronger, compared with

wt-hTRβ1; whereas, wt-hTRβ1 and m-hTRβ1 expression had no significant effect on proliferation in the absence of T3.

To understand how wt-hTRβ1 and m-hTRβ1 overexpression inhibits cancer cell proliferation *in vitro*, flow cytometry was conducted to evaluate apoptosis in MDA-MB-468 cells transfected with pcDNA3.1/NNSCS, pcDNA3.1-wt-hTRβ1/NNSCS and pcDNA3.1-m-hTRβ1/NNSCS. wt-hTRβ1 and m-hTRβ1 enhanced apoptosis in the presence of 10 nM T3, and the effects of m-hTRβ1 were stronger, compared with wt-hTRβ1. By contrast, wt-hTRβ1 and m-hTRβ1 expression had no significant effect on apoptosis in the absence of T3 (Fig. 3B and C).

Effects of hTRβ1 on Bak, Bax and Caspase-3 expression in MDA-MB-468 cells. wt-hTRβ1 and m-hTRβ1 reduced cell proliferation mainly through the promotion of apoptosis (Fig. 3). To determine the possible pathways that wt-hTRβ1 and m-hTRβ1 act to promote apoptosis, the relative expression levels of key apoptotic regulators, including *Bak*, *Bax* and *Caspase-3*, were detected in the different transfection groups.

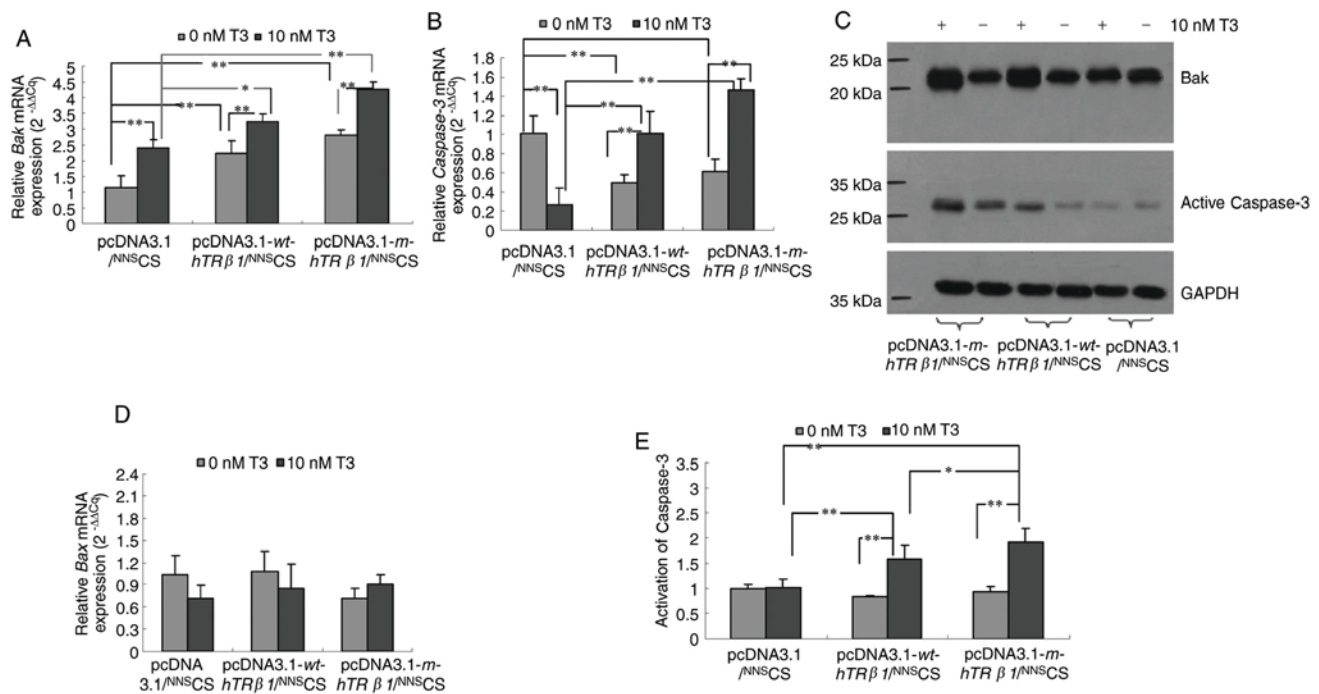


Figure 4. Expression of *Bak*, *Bax* and *Caspase-3* and activity of *Caspase-3* in MDA-MB-468 cells. Reverse transcription-quantitative polymerase chain reaction analysis of the relative gene expression of (A) *Bak* and (B) *Caspase-3* in MDA-MB-468 cells following wt-hTR β 1 or m-hTR β 1-gene transfection. The expression levels were normalized to those of *GAPDH*; the relative expression level of each gene was calculated using $2^{-\Delta\Delta C_q}$. The data are presented as the mean \pm SD. (C) Western blot analysis of the effect of wt-hTR β 1 and m-hTR β 1 on the expression of *Bak* and *Caspase-3*. The results are from a single experiment that is representative of three independent experiments. (D) Reverse transcription-quantitative polymerase chain reaction analysis of the relative gene expression of *Bax* in MDA-MB-468 cells following wt-hTR β 1 or m-hTR β 1-gene transfection. (E) Colorimetric assay of *Caspase-3* activation. Data are presented as the mean \pm SD. * P <0.05; ** P <0.01. wt-hTR β 1, wild-type human TR β 1; m-hTR β 1, modified human TR β 1; NNS, nucleic kinase substrate short peptide; CS, chitosan; T3, triiodothyronine; SD, standard deviation.

Expression of wt-hTR β 1 and m-hTR β 1 resulted in a gradual but significant upregulation of *Bak* and *Caspase-3* mRNA and protein expression in the presence of T3. However, wt-hTR β 1 and m-hTR β 1 expression downregulated *Caspase-3* gene expression in the absence of T3 (Fig. 4A-C). Expression of wt-hTR β 1 and m-hTR β 1 had no effect on *Bax* mRNA expression regardless of T3 presence or absence (Fig. 4D).

hTRβ1 increases the activity of Caspase-3 in MDA-MB-468 cells. As the expression of wt-hTR β 1 and m-hTR β 1 upregulated *Caspase-3* expression in MDA-MB-468 cells in the presence of T3 (Fig. 4B and D), *Caspase-3* activity was evaluated in these cells. Fig. 4E depicted that the expression of wt-hTR β 1 and m-hTR β 1 also increased the activity of *Caspase-3* in the presence of T3. Increased *Caspase-3* activity is indicative of elevated apoptotic activity (17), and elevated *Bak* promotes apoptosis via the activation of caspases (18). These data indicated that wt-hTR β 1 and m-hTR β 1 can increase the expression of *Bak* to promote apoptosis through the activation of *Caspase-3*. In addition, the effect of m-hTR β 1 is stronger, compared with wt-hTR β 1.

Discussion

TRs can function as a tumor suppressor (5,10). Evidence from *in vivo* and *in vitro* studies supports the tumor-suppressive function of TR β 1 in breast cancer (1,19,20). Nonetheless, controversial conclusions are abundant regarding the role of thyroid hormones and their receptor TR β 1 in the occurrence

and development of breast cancer (21). Thyroid hormones and breast cancer and normal mammary glands (1). Decreased expression, promoter hypermethylation or expression of truncated TR β 1 has been observed in human breast cancer (22), and truncated TR β 1 has been associated with cancer occurrence (23). rTR β Δ is an extended receptor (11), and to explore the association between the extended receptor rTR β Δ and cancer, TR β Δ was overexpressed in rat breast cancer SHZ-88 cells *in vitro*. The results demonstrated that rTR β Δ clearly inhibited proliferation of SHZ-88 cells. Although rTR β Δ is highly homologous to rTR β 1, the former contains a larger DBD (Fig. 1A); thus, the strong inhibitory effect of rTR β Δ on breast cancer cells may be closely associated with this additional 108 bp. Sequence analysis has revealed high conservation between the DBD region of rats and humans (Fig. 1A). Accordingly, this extra 108-bp exon from the DBD of rTR β Δ was introduced into the corresponding location of wt-hTR β 1 by site-directed mutagenesis, but did not change the original reading frame of wt-hTR β 1 (Fig. 1B and C).

wt-hTR β 1 contains an 'EG...G' P-box sequence in the DBD (24), and studies have indicated that the first two amino acids (EG) of the P-box are the most crucial for TRE binding specificity (25,26). If the first two amino acids are mutated, TR β binding ability to TRE is significantly reduced or completely lost, though the requirements of the third amino acid are not strict (24,27). The additional 36 amino acids were introduced to the C-terminal EG of the P-box, and the third amino acid was changed from G to P (Fig. 1A). In the present study, the aim was to clarify whether artificial m-hTR β 1 is a

functional receptor and to evaluate the effect of m-hTR β 1 on MDA-MB-468 cells.

Overall, the results of the EMSA and transcriptional activity analysis demonstrated that m-hTR β 1 can bind TREs (DR4) with transactivation by T3. An *in vitro* analysis demonstrated that the overexpression of m-hTR β 1 in MDA-MB-468 cells could inhibit their proliferation via the promotion of apoptosis in the presence of T3; the effect of m-hTR β 1 was stronger, compared with wt-hTR β 1. One study has demonstrated that the levels of the thyroid hormone T3 are elevated in the serum of patients with breast cancer (28), where this hormone promotes the growth and motility of breast cancer cells (18,29,30). However, since Beatson (31) described the treatment of metastatic breast tumors with thyroid extracts, a number of *in vitro* and *in vivo* studies have demonstrated that T3 can inhibit breast tumor cell proliferation and metastasis (1,32). These data indicated that thyroid hormones may have a bidirectional effect on breast cancer cells, particularly among different breast cancer cell lines. The results indicated that T3 inhibits the proliferation of MDA-MB-468 cells that overexpress m-hTR β 1.

Previous studies have demonstrated that TR β expression reduces MCF-7 tumor growth through the activation of apoptosis *in vivo* (20) and the induction of apoptosis in human breast cancer MCF-7 cells in the presence of T3 *in vitro* (1). To explore the underlying mechanism that m-hTR β 1 promotes MDA-MB-468 cell apoptosis, the expression of pro-apoptotic *Caspase-3*, *Bax* and *Bak*, was detected and the Caspase-3 activity was examined. TR β expression induces apoptosis in breast cancer cells via an increase in cleaved PARP and Caspase-3 (20,33). In the present study, it was determined that in the presence of T3, the overexpression m-hTR β 1 promoted MDA-MB-468 cell apoptosis via the upregulation of *Caspase-3* and *Bak* mRNA and protein expression and via an increase in Caspase-3 activity. In addition, the pro-apoptotic effect of m-hTR β 1 was stronger, compared with wt-hTR β 1. These results indicated that modification of the DBD region may alter the TR β activation intensity of target genes.

To conclude, an artificial *m-hTR β 1* was constructed through the introduction of a novel 108-bp exon into the DBD of *wt-hTR β 1*. m-hTR β 1 was functional in MDA-MB-468 cells and inhibited MDA-MB-468 cell proliferation, as it promoted apoptosis in the presence of T3. In the future, the role of m-hTR β 1 in other cancer cell lines *in vitro* and its role in tumorigenesis *in vivo*, using xenograft models, will be ascertained. This information may provide a novel possibility of gene therapy for TR-deficient breast cancer. Therefore, inhibition of the growth of cancer cells (TR-deficient) and improvements in patient survival may be possible via TR transgenes.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

RLZ designed the project, and contributed to all experiments, analysis of data and to writing the manuscript. XXP contributed to all experiments and to writing the manuscript. YYZ, YLS, LJW, WS and QL conducted the experiments. All authors read and provided their approval for the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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