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Tristetraprolin suppresses AHRR expression through mRNA destabilization

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ABSTRACT

The aryl hydrocarbon receptor repressor (AHRR) inhibits the transcription of the aryl hydrocarbon receptor (AHR) by binding to XRE. We report that AHRR expression is inhibited by tristetraprolin (TTP), an AU-rich element (ARE)-binding protein. Overexpression of TTP decreased the mRNA stability and protein expression of AHRR, and TTP-overexpressing cells made smaller colonies than the control. Contrarily, inhibition of TTP by siRNA increased AHRR expression. Analyses of point mutants of the AREs demonstrated that AREs were responsible for the TTP-mediated destabilization of AHRR mRNA. RNA EMSA revealed that TTP directly binds to the AHRR 3′UTR. Taken together, we demonstrate that TTP acts as a negative regulator of AHRR and may affect tumor development through induction of tumor suppressor genes as observed in MDA-MB435.

1. Introduction

The aryl hydrocarbon receptor repressor (AHRR) is a member of the basic-region helix-loop-helix/PER-Arnt-SIM (bHLH/PAS) protein family and represses the transcriptional activity of AHR (aryl hydrocarbon receptor) by competing heterodimer formation with the AHR nuclear translocator (ARNT) and binding to the xenobiotic response element (XRE) [1]. The expression of AHRR in tissues has been reported in several studies [2,3]. The levels of AHRR mRNA expression is high in the lung, ovary, spleen, and pancreas, however, it is poorly expressed in the small intestine, liver, kidney, colon and heart.

The AHR is a ligand-activated transcription factor that mediates a pleiotropic response to various environmental toxins [4], such as benzo[a]pyrene, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [5]. The AHR pathway regulates various physiologic mechanisms, including drug metabolism, xenobiotic detoxification, teratogenesis, immunosuppression and tumor promotion [6–8]. Moreover, there is evidence that the AHR pathway is implicated in promoting tumors from different anatomical origins [9,10]. In recent studies, AHR was reported as a tumor suppressor from the observation that AHR-knockout mice have inhibited prostate and liver carcinogenesis [11,12].

Post-transcriptional regulation of gene expression is mediated by AREs located in the 3′-untranslated region (3′UTR) of a variety of short-lived mRNAs such as cytokines and proto-oncogenes [13]. The destabilizing mechanism of AREs is mediated by various ARE binding proteins [14]. Tristetraprolin (TTP), a well-characterized ARE-binding protein, accelerates degradation of a number of ARE-containing transcripts [15] and may control cancer cell growth [16,17]. We have investigated if low expression of TTP correlates with increased expression of proto-oncogenes in various cancers in recent reports [18–22].

In the present study, we investigated the role of TTP in the regulation of AHRR gene expression in a human breast cancer cell line. Our data demonstrate that overexpression of TTP decreased AHRR expression in MDA-MB435 cells. On the other hand, inhibition of TTP by siRNA increased AHRR expression. AHRR mRNA contains two AREs in the 3′UTR and TTP promoted the decay of AHRR mRNA through direct binding to the 3′UTR of AHRR. Our results show that AHRR mRNA is a physiological target of TTP and suggest that TTP regulation of AHRR transcript stability may modulate the effect of this protein on cell proliferation.

2. Materials and methods

2.1. Cells

Human breast cancer cell lines MCF7, ZR-75-1, MDA-MB231 and MDA-MB435 were purchased from the American Type Culture

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Collection (Manassas, VA) and were maintained in RPMI 1640 media supplemented with 10% FBS (HyClone, Rogan, UT) at 37 °C in a humidified atmosphere of 5% CO_2.

For the MTT cell proliferation assay, cells were plated in triplicate at 1×10^4 cells/well in 96-well culture plates in RPMI1640. At the indicated times, CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI) was added to each well according to the manufacturer's instructions and absorbance at 490 nm (OD490) was determined for each well using a Wallac Vector 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

For soft agar cologenic assay, cells were resuspended in 3 ml of RPMI1640 containing 10% FBS and 0.35% agarose and plated in 6 cm dishes with 3 ml of pre-solidified RPMI1640 in 0.7% agar containing 10% FBS. Dishes were incubated at 37 °C for 2 weeks, and colonies larger than 0.1 mm in diameter were counted.

2.2. Plasmids and siRNAs

The pcDNA6/V5-TTP construct has been described previously [20]. Two oligonucleotides containing ATTTA motifs of the AHRR mRNA 3′UTR were synthesized at Integrated DNA Technologies (Coralville, IA). The oligonucleotides sequences are as follows: Oligo-ARE-WT (TCGAGCATGCTGAGCATGGCACACTTCTGGCCTCTGGGC **ATTTA**TGG**ATTTA**AGACCAGGATGGTATTTCAGAAGCTTCCCACTTCCG C, GGCCGCGGAAGTGGGAAGCTTCTGAAATACCATCCTGGTCT**TAAAT** CCA**TAAAT**GCCCAGAGGCCAGAAGTGTGCCATGCTCAGCATGC).

Mutant oligonucleotides of ARE1 and ARE2 in which ATTTA pentamers were substituted with AGCGA were also synthesized. The oligonucleotides were ligated into the *Xho*I/*Not*I site of the psiCHECK2 Renilla/firefly dual-luciferase expression vector (Promega, Madison, WI).siRNAs against human TTP (TTP-siRNA) (siTTP) and control siRNA (scRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Transfections and luciferase assay

Cells (5×10^6) were transfected with the various plasmid constructs using Fugene6HD (Promega, Madison, WI) and with siRNAs using jetPRIME (Polyplus, NY) according to the manufacturer's instructions.

Lysates of the transfected cells were mixed with luciferase assay reagent (Promega, Madison, WI) and the chemiluminescent signal was measured in a Wallac Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland). Renilla luciferase activity of psiCHECK2/AHRR-ARE-WT was normalized to firefly luciferase in each sample.

2.4. Electrophoretic Mobility Shift Assay (EMSA)

The biotinylated RNA probes for wild-type (Oligo-ARE-WT, 5′ CCUGUAUUUAUUUG-AGCUAUUUAAGGAUCUAUUUAUGUUUAAG-UAUUUAGAAAA-3′) and mutant (Oligo-ARE-MUT, 5′CCUGUAGCAU UUGAGCUAGCGAAGGAUCUAGCAUGUUUAAGUAGCGAGAAAA-3′) were synthesized by Samchully Pharm. Co., Ltd. (Seoul, Korea). Cytoplasmic extracts were prepared from MDA-MB435 cells using NE-PER® Nuclear and Cytoplasmic extraction Reagent (Thermo Pierce Biotechnology Scientific, Rockford, IL). RNA EMSA was performed using the Lightshift® Chemoluminescent EMSA Kit (Thermo Pierce Biotechnology Scientific, Rockford, IL) according to the manufacturer's instructions. Briefly, 10 fmol of biotinylated RNA was combined with 4 μg of cytoplasmic protein from cells in a binding buffer. For the supershift EMSA, anti-TTP antibody (ab36558, Abcam, Cambridge, MA) or control antibody (Sigma, Louis, MO) was added to the reaction mixture. After the addition of antibodies, reaction mixtures were incubated overnight on ice. The reaction mixtures were resolved on 5% non-denaturing polyacrylamide gels in 0.5× Tris borate/EDTA buffer. Gels were transferred to nylon membrane (Hybond™-N^+; GE Healthcare Bio-Sciences Corp. Piscataway, NJ) in 0.5× Tris Borate/EDTA at 100 V and 4 °C for 1 h.

The RNAs were cross-linked to the membrane and detected using streptavidin-horseradish peroxidase binding and chemiluminescence.

2.5. SDS–PAGE analysis and immunoblotting

Proteins were resolved by SDS–PAGE, transferred to Hybond-P membranes (GE Healthcare Bio-Sciences Corp. Piscataway, NJ), and probed with the appropriate dilution of anti-TTP antibody (Sigma, Louis, MO, 1:3000) and anti-AHRR antibody (Abcam, Cambridge, MA, 1:3000). Immunoreactivity was detected using the ECL detection system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Films were exposed at multiple time points to ensure that images were not saturated.

2.6. Quantitative real-time PCR

For RNA kinetic analysis, we used Actinomycin D and assessed AHRR mRNA expression by quantitative real-time PCR. Quantitative real-time PCR was performed using the BioRad CFX96 Optics Module (BioRad, CA) by monitoring the increase in fluorescence of the SYBR Green dye (iQ SYBR green supermix, Biorad, CA). Specificity of each primer pair was confirmed by melting curve analysis and agarose gel electrophoresis. PCR primer pairs were as follows: TTP: CGCTACAAGACTGAGCTAT, GAGGTAGAACTTGTGACAGA; AH RR: CTTAATGGCTTTGCTCTGGTCG, TGCATTACATCCGTCTGATGGA.

2.7. Statistics

For statistical comparisons, *P*-values were determined using the Student's *t*-test.

3. Results

3.1. Overexpression of TTP reduces the expression level of AHRR and enhances the decay of AHRR mRNA in MDA-MB435 cells

TTP is involved in the degradation of a number of genes required for cell growth and proliferation which contain an ARE within their 3′UTR [23,24]. Analysis of the human AHRR mRNA 3′UTR revealed the presence of two ARE motifs. To determine whether TTP expression is reciprocally related with AHRR expression in human breast cancer cell lines, we analyzed the expression of TTP and AHRR in four human breast cancer cell lines. Cell lines with high TTP expression levels (MCF7 and ZR-75-1) showed relatively low expression of AHRR, hence cells (MDA-MB231 and MDA-MB435) with low TTP expression levels showed relatively high level of AHRR expression (Fig. 1A), suggesting a inverse correlation between TTP expression and AHRR expression in human breast cancer cell lines. We next examined whether TTP affects the expression of AHRR; we transiently transfected MDA-MB435 cells with pcDNA6/V5-TTP (MDA-MB435/TTP). As a negative control, MDA-MB435 cells were transfected with an empty pcDNA6/V5 vector (MDA-MB435/pcDNA). The expression levels of AHRR were checked by RT-PCR and Western blot analysis (Fig. 1B). Our results showed that the overexpression of TTP significantly reduced the expression of AHRR.

To test whether the down-regulation of TTP effects AHRR expression, we used siRNA against TTP to reduce the expression level of TTP in MCF7 cells, which most highly expressed TTP among the four human breast cancer cell lines. Down-regulation of TTP by treatment with siTTP significantly increased the expression level of AHRR (Fig. 1C). However, treatment with non-specific siRNA