Thyroid hormone receptor β1 suppresses proliferation and migration by inhibiting PI3K/Akt signaling in human colorectal cancer cells

LEI ZHU1, GUANGANG TIAN1*, QIN YANG1, GEJING DE2, ZHIGANG ZHANG1, YAHUI WANG1, HUIZHEN NIE1, YANLI ZHANG1, XIAOMEI YANG1 and JUN LI1

1State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200240; 2Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100050, P.R. China

Received February 19, 2016; Accepted April 18, 2016

DOI: 10.3892/or.2016.4931

Abstract. Thyroid hormone receptor β1 (TRβ1) is a ligand-dependent transcription factor that belongs to the superfamily of nuclear receptors. TRβ1 has been found to act as a tumor suppressor in many solid tumors including breast cancer and hepatocellular carcinoma, but its role in the progression of human colorectal cancer (CRC) remains unclear. In this study, microarray data analysis revealed that TRβ1 mRNA was downregulated in CRC tumors compared with that in the normal counterparts in both The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets. Using a CRC tissue microarray (TMA), we confirmed that the expression of TRβ1 was decreased in human CRC tumor tissues in contrast to normal colorectal mucosal tissues. Notably, the TRβ1 expression was strongly correlated with tumor size (p=0.045). Furthermore, we found that CRC cell proliferation and migration were significantly inhibited by TRβ1 overexpression in vitro. Mechanistic studies indicated that activated phosphorylated Akt was clearly suppressed by TRβ1 in the CRC tissues and cells. In conclusion, this study provides evidence that TRβ1 plays a critical role in the progression of CRC via the PI3K/Akt pathway, and the TRβ1 gene may represent a novel target for CRC therapeutics.

Introduction

Colorectal cancer (CRC) is the third most common cancer in males and the second in females; it also remains the third leading cause of cancer-related deaths (1). Innovative therapeutic strategies have greatly improved the long-term survival of CRC patients over the past decade; however, a significant proportion of patients suffer from drug resistance, relapse and poor outcomes (2,3). The development of CRC is a complex process involving a series of genetic and epigenetic alterations (4). Thus, understanding the molecular mechanisms of CRC carcinogenesis and progression are extremely crucial to improve CRC diagnosis and treatment.

Thyroid hormones [such as triiodothyroine (T3)] regulate the growth, development and differentiation in vertebrates (5). T3 binds to specific high affinity receptors [thyroid receptors (TRs)] which belong to the superfamily of nuclear receptors (6). They function as ligand-modulated transcription factors by binding to thyroid hormone response elements (TREs) located in the promoter regions of target genes (7). Two human TR genes, TRα and TRβ, respectively, are located on human chromosomes 17 and 3. By alternative splicing and different promoter usage, these two genes yield at least four proteins: thyroid hormone receptor α1 (TRα1), TRα2, thyroid hormone receptor β1 (TRβ1), and TRβ2 (8). Most tissues of the human body express TRs, but there is differential expression of the TR isoforms. TRα1 predominates in skeletal muscle and brown fat, TRα2 in brain, TRβ1 in brain, liver and kidney, and TRβ2 in the central nervous system and developing retina (9-11).

Aberrant expression or mutation of TRs are common events in human cancer (12). Somatic mutation of TRs have been found in human hepatocellular carcinoma (13), renal clear cell carcinoma (14,15), breast cancer (16), pituitary tumors (17,18) and thyroid cancer (19). Moreover, an increasing number of studies indicate that TRs are potent suppressors of tumorigenesis, invasiveness, and metastasis (20). Mice devoid of functional TRs (TRα1−/−, TRβ1−/−) spontaneously develop follicular thyroid cancer and metastasis to the lung (21). In hepatocarcinoma cells transfected with TR, T3 was found to downregulate expression of pituitary tumor transforming gene 1 (PTTG1) and inhibit cell growth (22). Martinez-Iglesias et al. identified that expression of TRβ1 in hepatocarcinoma and breast cancer cells reduced tumor growth and caused partial
mesenchymal-to-epithelial cell transition (20). These findings suggest that TRs may act as tumor suppressors and constitute novel therapeutic targets in cancer.

In this study, we report that TRβ1 expression was decreased in human CRC tissues compared to that in normal controls. In two CRC cell lines overexpressing TRβ1, the cell proliferation and migration was suppressed. The PI3K/Akt signaling pathway plays an important role in tumor progression. It has been reported that Akt is over-activated in thyroid cancer in humans and in mice carrying mutated TRβ genes (23,24). Here, we identified that PI3K/Akt signaling was inactivated by TRβ1 expression in CRC tissues and cells.

Materials and methods

Clinical samples. A total of 222 CRC tissue sections and adjacent normal colorectal mucosal tissues were collected from January 2008 to December 2010 at Renji Hospital, School of Medicine, Shanghai Jiao Tong University. All of the samples were formalin-fixed and paraffin-embedded. Important clinical data, such as tumor size, lymphatic metastasis, histological grade were collected from the medical records of 100 patients. In addition, 29 pairs of fresh CRC and adjacent specimens were available and snap-frozen in liquid nitrogen immediately after surgery and stored at -80˚C until use. All of the human materials were obtained with informed approval of the World Health Organization Collaborating Center for Research in Human Production (authorized by the Shanghai Municipal Government).

Cell culture. Two human CRC cell lines HCT116 (ATCC CCL-247) and SW620 (ATCC CCL-228) were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China), and separately cultured in RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics (both from Gibco, Grand Island, NY, USA) at 37˚C in a humidified incubator under 5% CO2 condition.

Tissue microarray (TMA) and immunohistochemistry (IHC) staining. TMA was constructed by Suzhou Xinxin Biotechnology Co., Ltd. (Suzhou, China). Cores with a 2-mm diameter were collected from individual paraffin-embedded sections and arranged in the recipient paraffin blocks. Then, 5-µm thick sections were placed on superfrost charged glass microscope slides.

Prepared slides were deparaffinized in xylene, and rehydrated in a series of graded ethanol. The antigens were retrieved in 0.01 M sodium citrate buffer (pH 6.0) using a microwave oven, and 3% hydrogen peroxide was used to block endogenous peroxidase activity. After washing for 60 min in PBS with 10% BSA to prevent non-specific binding, the tissue slides were incubated with the primary antibody for TRβ1 (Clone J51, 1:50 dilution; Santa Cruz Biotechnology, Inc., USA) or pAkt (Ser473) (#2118-1, 1:50 dilution; Abcam, USA) overnight at 4˚C. On the next day, the tissues were incubated with species-specific secondary antibodies (1:1,000; Abcam) for 60 min at room temperature. Immunostaining was carried out using a DAB substrate kit (Thermo Fisher Scientific, USA), followed by immersing into hematoxylin for nuclear counterstaining. A staining index was obtained as the intensity of positive staining (negative, 0; weak, 1; moderate, 2; or strong, 3 scores) and the proportion of immunopositive cells of interest was scored (<25%, 1; 25-50%, 2; >50-75%, 3; ≥75%, 4). All scores were subdivided according to the median values of the study cohort into two categories: low expression (< median) and high expression (≥ median).

Quantitative real-time PCR. Total RNA from the human tissues or CRC cells was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed through PrimeScript RT-PCR (Takara Bio, Inc., Shiga, Japan) according to the protocol. Real-time PCR analyses were performed with SYBR Premix Ex Taq (Takara Bio, Inc.) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) at the recommended thermal cycling settings: one initial cycle at 95˚C for 30 sec followed by 40 cycles of 5 sec at 95˚C and 31 sec at 60˚C. The primers were as follows: human TRβ1 forward, 5'-TTACAGCTTGGGAACACCG-3' and reverse, 5'-GCCGACATTCTCGGCACTGAT-3'; human β-actin forward, 5'-AGTTGCGTAAAACCCTTTGTG-3' and reverse, 5'-CACCTTTACCGTTCCAGTTTT-3'. The relative expression of TRβ1 was calculated and normalized using the 2-ΔΔCt method relative to β-actin. Independent experiments were conducted in triplicate.

Lentiviral overexpression. The lentiviral expression system was obtained from System Biosciences (SBI; Mountain View, CA, USA). The cDNA encoding human TRβ1 was amplified and cloned into pCDH-CMV-MCS-EF1-Puro, and then the expression vector was co-transfected with packaging vectors pSPAX and pMD2.G at a ratio of 3:2:1 into 293T cells using X-tremeGENE HP (Roche Diagnostics, GmbH, Mannheim, Germany). Lentiviruses were harvested at 48 and 72 h after transfection, and virus titers were determined. Target cells (1x104), including HCT116 and SW620 cells, were infected with 1x106 recombinant lentivirus-transducing units in the presence of 6 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA).

Dual-Luciferase reporter assay. TRE-activated firefly luciferase reporter vector (TRE-luc) was a kind gift from Dr Hao Ying at the Institute for Nutritional Sciences (SIBS), Chinese Academy of Sciences. The Renilla luciferase vector (pRL-TK) (Promega, Madison, WI, USA), driven by an HSV-TK promoter, was used as an internal control. HCT116 or SW620 cells were seeded in 96-well plates and co-transfected with TRE-luc (0.1 µg/well) and Renilla control plasmids (0.01 µg/well) following the manufacturer's instructions. After 48 h, the CRC cells were treated with 10 nM 3,3',5-triiodo-L-thyronine (T3) (Sigma-Aldrich) with dH2O as a control, and incubated for further 24 h. Luciferase activities were measured on a luminometer using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Cell viability and colony formation assay. Cells were seeded into a 96-well plate at 2,000 cells/well with 100 µl complete medium and cultured at 37˚C. A total of 10 µl Cell Counting
Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution was added to each well after 24, 48, 72, 96, and 120 h, respectively. After 1 h of incubation, WST-8 was metabolized to produce a colorimetric dye that is detected at OD450 nm by using a PowerWave XS microplate reader (BioTek Instruments, Inc.). The experiment was performed in triplicate and repeated twice. To determine clonogenic ability, the cells were seeded into a 6-well plate at 1,000 cells/well and cultured for 14 days. Cell colonies were then stained with crystal violet (Beyotime Institute of Biotechnology, Shanghai, China) and counted.

Migration assay. Cell migration assays were performed using Transwell chambers (BD Biosciences, Bedford, MA, USA). Cells (5x10^5) in 200 µl serum-free DMEM were seeded in the upper chamber and 800 µl medium supplemented with 10% FBS was added to the lower chamber. The migrated cells were fixed and stained with 0.1% (w/v) crystal violet 48 h later. Five randomly selected fields were photographed and the numbers were counted.

Western blot analysis. Cells were lysed in RIPA buffer containing 1 mM PMSF and protease inhibitor cocktail. The protein concentrations were measured by using a BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Then protein samples were separated on 10% SDS-PAGE gels under reducing condition and transferred onto nitrocellulose membranes (Millipore Corp., Billerica, MA, USA). After blocking in phosphate-buffered saline/Tween-20 (PBST) containing 1% BSA at room temperature for 1 h, the membranes were incubated overnight at 4°C with the primary antibodies. The following antibodies were used: anti-TRβ1 (1:500 dilution; Santa Cruz Biotechnology, Inc.), anti-Akt (#1081-1, 1:1,000 dilution), anti-phospho-Akt (Ser473) (#2118-1, 1:1,000 dilution) (both from Abcam), and anti-β-actin (1:10,000 dilution, Sigma-Aldrich). After washing with PBST, the membranes were incubated with species-specific secondary antibodies. Bound secondary antibodies were revealed by Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 16 (SPSS, Inc. Chicago, IL, USA). The Pearson's Chi-square test was used to analyze the relationship between TRβ1 expression and clinicopathological characteristics. The two-tailed Student's t-test was used for comparison between two groups. P<0.05 was considered to indicate a statistically significant result.

Results

TRβ1 expression is decreased in human CRCs. We firstly analyzed the normalized mRNA expression of TRβ1 from The
Cancer Genome Atlas (TCGA) data of 20 cancer types. TRβ1 expression was decreased in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ), the data of which were obtained from 41 and 9 pairs of tumor samples and normal tissues, respectively (Fig. 1A). To confirm the TRβ1 expression level in CRC, we analyzed two independent microarray datasets (GSE20916 and GSE9348) of healthy and CRC patients from the NCBI Gene Expression Omnibus (GEO) dataset-record. The results also showed that TRβ1 expression was significantly downregulated in the tumor specimens compared with that in the normal colorectal mucosal specimens in these two datasets (fold change was -4.169 and -5.858 respectively, Fig. 1B).

Furthermore, we measured the TRβ1 mRNA and protein levels in CRC tissues. Twenty-nine pairs of CRC and corresponding adjacent normal mucosal tissues were collected and subjected to quantitative real-time PCR. TRβ1 expression was decreased in 89.7% (26/29) of the CRC patients at the mRNA level, consistent with the data from the TCGA and GEO datasets (Fig. 2A). Using a CRC-TMA containing 222 pairs of CRC specimens and corresponding normal colorectal mucosal tissues, TRβ1 protein expression was detected by IHC staining. In normal mucosa, TRβ1 was localized predominantly in the nucleus and cytoplasm of surface epithelium cells, and it was weaker in the crypt bases (Fig. 2B). In carcinomas, TRβ1 expression was less prevalent (Fig. 2B), and it was downregulated in 70.10% (157/222) of the CRC patients (Fig. 2C).

These results suggest that attenuated expression of TRβ1 may contribute to CRC carcinogenesis and progression.

**Association between TRβ1 expression and the clinicopathological features of CRC.** The basic clinical characteristics of 100 CRC patients are summarized in Table I. The Chi-square test was used to analyze correlations between TRβ1 protein expression and clinicopathological parameters in the CRC cases. The results indicated that TRβ1 expression was significantly correlated with tumor size (p=0.045). No
Overexpression of TRβ1 inhibits CRC cell proliferation. To identify the function of TRβ1 in CRC, we established stable cell lines by a lentivirus carrying the TRβ1 gene in HCT116 and SW620 cells, which exhibit a low endogenous level of TRβ1. Stable HCT116 and SW620 cells transfected with an empty vector were used as controls. TRβ1 was overexpressed in the lenti-TRβ1-infected cells as characterized both by quantitative RT-PCR and western blot analysis (Fig. 3A and B). To ensure that ectopic TRβ1 was capable to drive gene transcription, we used a luciferase reporter vector containing TRE. The TRE-driven luciferase expression readily responded to T3 in both the HCT116 and SW620 cells stably overexpressing TRβ1 (Fig. 3C).

CCK-8 assay was performed to verify the role of TRβ1 in the two stable CRC cell lines. Compared with the control cells, overexpression of TRβ1 significantly inhibited the proliferation of the HCT116 and SW620 cells stably overexpressing TRβ1 (Fig. 4A). Consistently, TRβ1 suppressed the colony formation of CRC cells (Fig. 4B).

Overexpression of TRβ1 suppresses the migration of CRC cells. To further evaluate the effect of TRβ1 on CRC metastasis, we used Transwell assay. Our findings showed that the number of migrated cells in the TRβ1-overexpressing group was clearly decreased compared with the number in the control group (Fig. 4C).

Table I. Correlation of TRβ1 expression with clinicopathological characteristics of the 100 colorectal cancer patients.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Expression of TRβ1</th>
<th>P-value (χ² test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Low</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>56</td>
<td>39</td>
</tr>
<tr>
<td>Female</td>
<td>44</td>
<td>33</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>&gt;60</td>
<td>72</td>
<td>54</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>67</td>
<td>44</td>
</tr>
<tr>
<td>&gt;5</td>
<td>33</td>
<td>28</td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>33</td>
<td>26</td>
</tr>
<tr>
<td>No</td>
<td>67</td>
<td>46</td>
</tr>
<tr>
<td>Histological grade (WHO)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>G2</td>
<td>80</td>
<td>57</td>
</tr>
<tr>
<td>G3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>P53 staining</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>No</td>
<td>62</td>
<td>42</td>
</tr>
</tbody>
</table>

Bold implies statistical significance, p<0.05. TRβ1, thyroid hormone receptor β1.

Overexpression of TRβ1 inhibits PI3K/Akt signaling in CRC cells. To elucidate the underlying mechanism of TRβ1-suppressed CRC cell proliferation and migration, we examined
whether PI3K/Akt, which plays an important role in tumor progression (25), was involved in the function of TRβ1 in CRC cells. As shown in Fig. 5A and B, overexpression of TRβ1 led to significant decreases in the level of phosphorylated Akt with no change in total Akt expression in both the HCT116 and SW620 cells. When TRβ1-expressing HCT116 cells were treated with T3, the decrease in pAkt expression was more obvious (Fig. 5C).

Furthermore, the protein expression level of phosphorylated Akt was detected by IHC from the CRC-TMA. We found that phospho-Akt expression was inversely correlated with TRβ1 expression in 170 CRC tissues (R=-0.166, p=0.03, Fig. 5D and E). This result suggests a possible mechanism - TRβ1 inhibits CRC cell proliferation and migration by suppressing PI3K/Akt signaling.

Taken together, our results demonstrated that overexpression of TRβ1 suppressed the activation of the PI3K/Akt signaling pathway and inhibited the proliferation and migration of CRC cells (Fig. 5F).

Discussion

In the present study, we found that TRβ1 mRNA and protein were downregulated in cancer tissues compared with the levels in their corresponding normal tissues as detected by qRT-PCR and IHC staining, and TRβ1 expression was significantly correlated with tumor size. In vitro cellular experiments demonstrated that overexpression of TRβ1 inhibited the proliferation and migration of CRC cells. Moreover, the Akt signaling pathway was suppressed by ectopic TRβ1 in the CRC tissues and cells. All our data suggest that TRβ1 acts as a tumor suppressor in CRC progression. For the first time to the best of our knowledge, the functional and clinical significance of TRβ1 expression was studied in CRC.
Aberrant expression or mutations of TRs are common events in human cancer, and TRs also have an important role in tumor progression in cancer cell lines (20,22) and experimental animal models (26–28), suggesting that these receptors may be involved in human cancer. Horkko et al. reported that TRβ1 is always present in normal colorectal mucosal epithelium but less frequent in CRC, and its expression is associated with the presence of K-ras mutations. However, the role of TRβ1 in the progression of CRC remains unknown. In this study, the TRβ1 expression pattern was analyzed in datasets (TCGA, GSE20916 and GSE9348) and in human tissues by qRT-PCR and IHC. Consistent with the findings of Horkko et al. (30), we confirmed that TRβ1 expression was decreased in the CRC tissues compared with normal mucosal tissues. Further study revealed that tumor size was closely correlated with TRβ1 expression. Yet, more clinical samples and data are needed to identify the association between TRβ1 and overall survival (OS) and disease-free survival (DFS).

Furthermore, the biological functions of TRβ1 in CRC were detected by cell viability and migration assays. We found that overexpression of TRβ1 significantly suppressed CRC cell proliferation and migration. Yet, the in vivo role of TRβ1 in CRC carcinogenesis remains undetermined to date. CRC
mouse models induced by genetic alterations (e.g., APCmin/mice) or by chemical carcinogens are needed to reveal the pathological role of TRβ1 in colonic carcinogenesis.

The PI3K/Akt signaling pathway modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression and cellular growth, and components of this pathway are frequently altered in human cancers. In the present study, we found that overexpression of TRβ1 resulted in obvious decreased activation of Akt phosphorylation. However, it remains unconfirmed whether the effect of TRβ1 on cell survival and migration are mediated by the PI3K/Akt pathway. Moreover, TRs physically interact with the regulatory p85α subunit of PI3K to modulate downstream signaling pathway in thyroid cancer (21, 29). Whether or not TRβ1 inhibits Akt phosphorylation by direct interaction with PI3K in CRC remains unknown. TRβ1 also acts as a transcriptional factor and may regulate a series of target genes in CRC cells. Which target gene is involved in the suppression of cell viability and migration by TRβ1, and whether or not these target genes regulate CRC progression via the PI3K/Akt signaling pathway require further investigation.

In summary, this is the first study to report that TRβ1 plays a critical role in CRC by regulating the PI3K/Akt pathway. Future functional experiments with CRC mouse models are needed to clarify the role of TRβ1 in CRC progression and metastasis. Although the detailed mechanism remains to be delineated, this preliminary study provides further knowledge of the biological functions of TRβ1 in CRC and suggests that TRβ1 could be a potential target for CRC therapeutics.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (81472678), the Shanghai Natural Science Foundation (13ZR1440100) and the State Key Laboratory of Oncogenes and Related Genes (91-1511). We thank Dr Hao Ying for providing the luciferase reporter vector.

References