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Stimulation of Interferon-Stimulated Gene 20 by Thyroid Hormone Enhances Angiogenesis in Liver Cancer

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Abstract

Thyroid hormone, 3,3′,5-triiodo-L-thyronine (T_3), mediates several physiological processes, including embryonic development, cellular differentiation and cell proliferation, via binding to its nuclear thyroid receptors (TR). Previous microarray and Chromatin immunoprecipitation (ChIP)-on-ChIP analyses have revealed that interferon-stimulated gene 20 kDa (ISG20), an exoribonuclease involved in the antiviral function of interferon, is up-regulated by T_3 in HepG2-TR cells. However, the underlying mechanisms of ISG20 action in tumor progression remain unknown to date. Here, we verified induction of ISG20 mRNA and protein expression by T_3 in HepG2-TR cells. Based on the ChIP-on-ChIP database, potential thyroid hormone responsive element of the ISG20 promoter region was predicted, and the result confirmed with the ChIP assay. Functional assays showed that forced expression of ISG20 leads to significant promotion of metastasis and angiogenesis, both *in vitro* and *in vivo*. Furthermore, the angiogenic-related protein, interleukin-8 (IL-8), was up-regulated through a T_3-mediated increase in ISG20, as determined using a human angiogenesis array kit. Induction of IL-8 signaling activated the p-JAK2/p-STAT3 pathway, in turn, leading to promotion of tumor metastasis and angiogenesis. Furthermore, ISG20 overexpression in hepatocellular carcinoma (HCC) specimens was positively correlated with clinical parameters, including vascular invasion, α-fetoprotein and tumor size. Higher ISG20 expression was significantly correlated with poorer recurrence-free survival in HCC patients. Our results collectively indicate higher TR-dependent expression of ISG20 in a subset of HCC, supporting an oncogenic role in HCC progression.

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Abbreviations: AR, Amphiregulin; ChIP, Chromatin immunoprecipitation; CM, Conditional medium; EMT, Epithelial-mesenchymal transition; Endostatin, Collagen XVIII; HCC, Hepatocellular carcinoma; HUVEC, Human umbilical vein endothelial cell; IGFBP-3, Insulin-like growth factor binding protein-3; IL-8, Interleukin-8; IHC, Immunohistochemistry; ISG20, Interferon-stimulated gene 20 kDa; T_3, Thyroid hormone; TRE, Thyroid hormone response element; TF, Coagulation factor III; TSP-1, Thrombospondin-1; TSS, Transcription start site; TR, Thyroid receptors; RFS, Recurrence-free survival; VEGF, Vascular endothelial growth factor.

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Introduction

The thyroid hormone (3,3′,5-triiodo-L-thyronin, T_3), is a central mediator of many physiological processes, including embryonic development, cellular differentiation, metabolism, and regulation of cell growth [1]. These T_3-regulated cellular processes are mediated via a genomic effect, requiring the activation of nuclear thyroid hormone receptors (TRs). Two major TR isoforms, TRα1 and TRβ1 have been identified, which are encoded on human chromosomes 17 and 3, respectively, and expressed differently in developing and adult tissues [2]. Tumor formation is usually caused by tumor cell growth and metastasis, depending on the capacity of tumor cells to recruit their own blood supply. Angiogenesis is essential for human cancer development [3]. The angiogenic process is balanced by the positive regulator, vascular endothelial growth factor (VEGF) [4–7] and the negative regulator, thrombospondin-1 (TSP-1) [8–10]. Previous studies have shown that thyroid hormone induces sprouting angiogenesis through the PDGF-Akt pathway in hypothyroid mice [11] and increases the activity of angiotensin II that supports neovascularization and stimulates endothelial cell motility [12]. Abundance evidence has demonstrated that thyroid status and disease affect tumor formation, growth and metastasis in experimental animals and humans [1]. The collective findings clearly indicate that the thyroid hormone and its receptor play tumor-promoting roles, although the underlying mechanisms of thyroid hormone-stimulated tumor angiogenesis remain to be established.

The interferon-stimulated gene product of 20 kDa, ISG20, has been identified from oligo microarray and ChIP-on-ChIP analyses. We examined the function of ISG20 involved in angiogenesis using MetaCore pathway analysis. ISG20 is a member of the 3′ to 5′ exonuclease superfamily that includes RNases with specificity for single-stranded RNA, and to a lesser extent, DNA [13–15], that possesses antiviral activity. Previous studies suggest that ISG20 participates in the cellular response to virus infection, and its antiviral activity is induced by NF-κB and IRF1 activation [16]. Furthermore, ISG20 promotes angiogenesis [17], although the underlying mechanism is unclear at present. In the current study, we focused on the mechanism underlying the angiogenesis-promoting activity of ISG20 stimulated by thyroid hormone in liver cancer.

Materials and Methods

Cell Culture and Preparation of T_3-Depleted Medium

The human hepatoma cell lines, HepG2 and SK-Hep1 were purchased from the American Type Culture Collection (Taipei, Taiwan). J7 cell line was obtained from Taiwan Hospital. These cells were authenticated by the Mission Biotech using the Promega StemElite™ ID system (Taiwan). These cells were routinely grown in DMEM supplemented with 10% fetal bovine serum. The HepG2 cell line used in this study was stably transfected with TRα (HepG2-TRα1) or TRβ (HepG2-TRβ1), with HepG2-neo as the control cell line. Serum was depleted of T_3 (Td) using resin [18]. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Establishment of ISG20-Overexpressing Cells and Determination of the Effects of IL-8 Depletion Using the Lentiviral System

Human IL-8 shRNA was purchased from the RNAi core center (Academia Sinica, Taiwan). IL-8 shRNA and ISG20 plasmid were cloned into the expression vector pLKO1. The plasmid and lentiviral packaging constructs, pCMV-ΔR8.91 and pMD.G, were cotransfected in Her-293T cells via TurboFect reagent Kit (Fermentas Life science, Waltham, MA). After 24-48 h, viral particles with ISG20 were collected to infect SK-Hep1, and viral particles with shIL-8 used to infect the SK-Hep1-ISG20-overexpressing cell line. After 48 h of incubation, cells were transferred to medium containing puromycin for selection.

Quantitative RT-PCR

HepG2-TRα1 and HepG2-TRβ1 cells were seeded into 10 cm diameter dishes and exposed to various treatments for the indicated times, prior to harvesting for RNA extraction. Total RNA was purified using TRIzol reagent (Life Technologies Inc., Carlsbad, CA) according to the supplier's protocol, and cDNA synthesized using a SuperscriptII kit (Life Technologies, Karlsruhe, Germany). Real-time PCR was conducted in 15 μl reaction mixtures containing 25 nM forward and reverse primers and 1×SYBR Green reaction mix (Applied Biosystems, Carlsbad, CA). All reactions were conducted in an ABI PRISM 7500 sequencer (Applied Biosystems, Foster City, CA).

Immunoblotting Analysis

Total cell lysates were analyzed via 8% to 10% (w/v) SDS-PAGE, and the separated proteins transferred to PVDF membranes. After washing in PBST (PBS containing 0.05% [v/v] Tween-20), blots were incubated in blocking solution (PBST with 5% [w/v] skimmed milk powder) containing primary antibody against ISG20, p-JAK2, JAK2 (GeneTex Inc., Irvine, CA), IL-8 (R&D Systems Inc., Minneapolis, MN), p-STAT3(Y705), STAT3 (Cell Signaling Technology Inc., Danvers, MA), VEGF-A, MMP-9, fascin or actin (Santa Cruz Biotechnology Inc., Dallas, TX) at 4°C overnight. After thorough washing, blots were further incubated with HRP-conjugated secondary antibody dissolved in blocking solution. Signals were detected via chemiluminescence using an ECL kit (Amersham) and recorded on X-ray film. JAK2 inhibitor and STAT3 inhibitor (S31-201) were from EMD Millipore (Darmstadt, Germany) and Merck (Canada).

Chromatin Immunoprecipitation (ChIP) Assay

HepG2-TRα1 cells treated with 10 nM T_3 for 24 h or left untreated were harvested and cross-linked with 1% formaldehyde for 10 min at room temperature in DMEM. Reactions were terminated with the addition of 0.125 M glycine. Subsequently, cell lysates were washed three times with PBS and resuspended in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 8.0), 0.1% SDS and 0.1% sodium deoxycholate) containing three protease inhibitors (1 mM PMSF, aprotinin, and leupeptin). Cell lysates were sonicated with a Misonix Sonicator 3000 Homogenizer (Mandel Scientific Company Inc., Guelph, ON, Canada) to disrupt chromatin. Sonicated DNA was between 200 and 1000 bp in length. Products were precleared with 60 μl protein A/G agarose (Sigma Chemicals, St. Louis, MO) for 2 h at 4°C. Complexes were immunoprecipitated with anti-TR and anti-IgG antibodies (R&D Systems, Inc., Minneapolis, MN). Enriched targets were hybridized to promoter microarrays (Welgene Biotech, ChIP-on-chip microarray) spanning –8 kb to +2 kb of the transcription start site (TSS) of 35 000 genes. The promoter fragments of target gene containing the TRE region were detected via q-RT-PCR.

Human Angiogenesis Array Kit

Cell culture supernatant was collected from ISG20-overexpressing and control of SK-Hep1 for 24 h. The angiogenic factors secreted in supernatant was measured using angiogenesis array kit (R&D systems, Minneapolis, MN), according to the manufacturer's instructions. After