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Original Articles

Histone deacetylase inhibitors upregulate Snail via Smad2/3 phosphorylation and stabilization of Snail to promote metastasis of hepatoma cells

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ABSTRACT

Hepatocellular carcinoma (HCC) remains the third most common cause of cancer-related mortality. Resection and transplantation are the only curative treatments available, but are greatly hampered by high recurrence rates. Histone deacetylase inhibitors (HDACIs) are considered to be promising anticancer agents in drug development. Currently, four HDACIs have been granted Food and Drug Administration (FDA) approval for cancer. HDACIs have shown significant efficacy in hematological malignancies. However, they have limited effects in epithelial cell-derived cancers, including HCC, and the mechanisms of these are not elucidated. In this study, our results demonstrated that HDACIs were able to induce epithelial-mesenchymal transitions (EMT) in hepatoma cells which are believed to trigger tumor cell invasion and metastasis. We found that HDACIs promoted the expression of Snail and Snail-induced EMT was critical for HDACI-initiated invasion and metastasis. We indicated that HDACIs upregulated Snail in two ways. Firstly, HDACIs upregulated Snail at the transcriptional level by promoting Smad2/3 phosphorylation and nuclear translocation, then combined with the promoter to activate the transcription of Snail. Secondly, we showed that HDACIs regulated the stabilization of Snail via upregulating the expression of COP9 signalosome 2 (CSN2), which combined with Snail and exposed its acetylation site, then promoted acetylation of Snail, thereby inhibiting its phosphorylation and ubiquitination to repress the degradation of Snail. All these results highlighted that HDACIs have limited effects in HCC, and the use of HDACIs combined with other targeted strategies to inhibit EMT, which explored in this study is a promising treatment method for treating HCC.

1. Introduction

Histone deacetylases (HDACs) are enzymes involved in remodeling of chromatin by deacetylating the lysine residue. They are known to act in parallel with tumor growth as they trigger the abnormal transcription of crucial genes that control essential cell functions, such as proliferation, cell cycle regulation, and apoptosis.

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The irregularity in HDAC function thus contributes to the initiation and progress of several tumors [1]. HDAC inhibitors (HDACIs) suppress histone deacetylation to regulate gene expression for increased levels of acetylated histones, induce chromatin relaxation, and alter gene expression, which target the growth stagnation, differentiation, apoptosis of tumor cells, and have less impact on normal cells [2,3]. HDACIs, which have a wide range of clinical applications, are emerging as a new class of anticancer agents with potent activity in inhibiting proliferation and inducing differentiation of numerous tumors [4–7]. In addition, we found that HDACIs can inhibit the expression of indoleamine 2,3-dioxygenase (IDO) induced by interferon (IFN)-γ, which could reverse IDO-mediated tumor immune tolerance and activate the immune response to tumors, the discoveries represent a new mechanism in the antitumor action of HDACIs and may have implications for development of clinical cancer immunotherapy [8,9]. HDACIs show good results and prospects for the treatment of tumors. Currently, four drugs, Vorinostat (SAHA), Romidepsin (FK-228), Belinostat (PXD-101), and Panobinostat (LBH-589) have been granted Food and Drug Administration (FDA) approval for cancer and several HDACIs are currently in various phases of clinical trials, either as monotherapy and/or in combination with existing/novel anticancer agents [10–17].

It is well established that HDACIs have shown significant efficacy in hematological malignancies. However, they have limited effects as monotherapy in the treatment of epithelial cell-derived cancers, including hepatocellular carcinoma (HCC) [18–21]. The detailed mechanism of this phenomenon has yet to be elucidated. This study demonstrated that HDACIs could induce epithelial-mesenchymal transitions (EMT) in hepatoma cells. EMT refers to the transfer of epithelial cells to mesenchymal cells in the particular physiological and pathological conditions. It was first recognized as a central differentiation process in early embryogenic morphogenesis [22]. In the EMT process, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility [23]. The downregulation of E-cadherin and upregulation of vimentin have been regarded as the markers of EMT [24,25]. As an important transcription factor, Snail can repress expression of E-cadherin and induce EMT in cancer cells, indicating that Snail plays a fundamental role in EMT and cancer metastasis [26–28]. In addition, overexpression of Snail correlates with tumor grade, nodal metastasis, and tumor recurrence and predicts a poor outcome in patients with various cancers [29,30]. All these discoveries highlight the importance of Snail in the initiation of EMT at metastasis, and EMT is considered an important contributor to the invasion and metastasis of epithelial-derived cancers [31,32]. We found that HDACIs can upregulate the expression of Snail. However, it remains unclear which extrinsic signals of HDACIs regulate the expression and activity of Snail and the induction of EMT at the tumor invasive front. Here, we have examined the regulation of Snail and its role in hepatoma cell migration, invasion, and metastasis mediated by HDACIs, which elucidates the reasons why HDACI monotherapy is unsatisfactory in clinical research of HCC and provides experimental support for the treatment of HCC with HDACIs combined with other strategies, including the targeted therapy to inhibit EMT explored in this study.

2. Materials and methods

2.1. Chemicals and reagents

Actinomycin D (Act D), sodium butyrate (NaB), and suberoylanilide hydroxamic acid (SAHA) were purchased from Sigma–Aldrich (Deisenhofen, Germany). pGL3-Snail and pcDNA-Snail were gifts from Sun Yat-sen University. pcDNA-3.1 (vector), pRL-TK, and dual-luciferase assay kits are products of Promega (Madison, WI, USA). The monoclonal anti-ubiquitin, anti-acetylation, anti-Snail, an-Smad2, anti-Smad3, anti-p-Smad2, anti-p-Smad3, anti-HA-Tag, anti-N-cadherin, anti-fibronectin, anti-GAPDH, and anti-Vimentin antibody, and the secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP) are products of Cell Signaling Technology (MA, USA). Antibodies for CSN2 and p-CSN2, were from BD Transduction (San Jose, CA). Protein A/G sepharose was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A Millicell chamber (8 μm) was purchased from Millipore (BD Biosciences, USA). SYBR Premix Ex Taq II is a product of TaKaRa BIO, Inc. (TBI, Japan). The secondary anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC), 4′,6-diamidino-2-phenylindole (DAPI) dye, double-stranded siRNA Snail, siRNA Smad2, siRNA Smad3, siRNA CSN2, and Lipofectamine 3000 were purchased from Invitrogen (Carlsbad, CA, USA). The Nuclear-Cytosol Extraction Kit was purchased from Pierce (USA).

2.2. Cell culture and mouse

The human hepatoma cell lines HepG2 and QGY-7703 were purchased from the American Type Culture Collection (ATCC). The two cell lines were used from May 2015 to December 2016. Both cell lines were authenticated by short tandem repeat analysis and passaged for fewer than 6 months before experiments. Vials were thawed and maintained in culture for only several weeks at a time. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated endotoxin-free fetal calf serum (FCS), 100 μg/ml of streptomycin, and 100 units/ml of penicillin in a humidified 5% CO2 atmosphere at 37 °C in an incubator. Nude mice were purchased from the Sun Yat-sen University (Guangzhou, China) Animal Center and raised under pathogen-free conditions. All animal studies were conducted in accordance with institutional guidelines for the care and use of experimental animals.

2.3. Cytotoxicity assay

The cytotoxicity of SAHA and NaB toward the cultured cells was assessed using MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assays (Sigma Chemical Co). Cells were seeded onto 96-well microplates at a density of 1×10^4 cells per well and incubated for 24 h. Cells were then treated with selected concentrations of SAHA and NaB for 24 h. Cells in culture medium alone served as the untreated control. The MTT reagent (5 mg/mL in distilled water) was prepared immediately prior to use. After removing the incubation medium from the wells, cells were washed with PBS, and 10 mL of MTT reagent was added. After incubation for 4 h at 37 °C, MTT reagent in 100 mL of dimethylsulfoxide (DMSO) was added to each well. Surviving cells were then detected by measuring absorbance at 570 nm using a plate reader. The cell viability was expressed as a percentage of the values obtained for the controls.

2.4. Western blotting analysis

Cells were lysed in cell lysis buffer, and lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Equal amounts of protein samples were separated on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with the primary antibody at a 1:1000 dilution overnight at 4 °C and then incubated with an HRP-conjugated secondary