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Standard CD44 modulates YAP1 through a positive feedback loop in hepatocellular carcinoma

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ABSTRACT

High expression levels of CD44 and YAP have been identified as poor prognostic factors in hepatocellular carcinoma (HCC). However, the mechanistic relationship between CD44 and YAP during HCC tumorigenesis remains largely unknown. To investigate the mutual regulation between standard CD44 (CD44S) and YAP1 in HCC cell lines and tissue samples, CD44S and YAP1 expression in 40 pairs of tumor samples and matched distal normal tissues from HCC patients was examined by immunohistochemical staining. High expression of either CD44S or YAP1 was associated with a younger age and worse pathology grade. In addition, high levels of CD44S and YAP1 were associated with increased vascular invasion and more severe liver cirrhosis, respectively. CD44S expression was positively correlated with YAP1 expression in these HCC tissues. In vitro experiments suggested that CD44S could positively regulate the expression of YAP1 and its target genes via the PI3K/Akt pathway in HCC cells. Moreover, CD44S is regulated by the YAP1/TEAD axis. These results reveal a novel positive feedback loop involving CD44S and YAP1, in which CD44S functions as both an upstream regulator and a downstream effector of YAP1 in HCC. This feedback loop might constitute a broadly conserved module for regulating cell proliferation and invasion during HCC tumorigenesis. Blocking this positive feedback loop that involves CD44S and YAP1 might represent a new approach for HCC treatment.

1. Introduction

The Hippo signaling pathway is an evolutionarily conserved signaling pathway that plays important roles in organ size control, tissue regeneration, and tumor suppression [1]. The transcriptional coactivator Yes-associated protein (YAP) is the major downstream effector of Hippo signaling. Briefly, when the Hippo pathway is activated in mammals, the phosphorylation cascade involving the Hippo core component kinases MST1/2 and Lats1/2 cooperates with the adaptor proteins Salvador 1 (SAV1) and MOB kinase activator 1A/B (MOB1a/b) to phosphorylate and inhibit YAP in the nucleus [2–4]. Activated Lats1/2 restrains YAP activity by phosphorylation at S127, which promotes YAP binding to 14-3-3 and leads to its cytoplasmic sequestration; the phosphorylated S127-YAP/14-3-3 complex subsequently undergoes cytoplasmic degradation [5]. In contrast, once this pathway is inactivated, dephosphorylated YAP localizes in the nucleus by interacting with TEA domain (TEAD) family transcription factors to induce the expression of target genes, such as connective tissue growth factor (CTGF), Cyr61, c-Myc, AXL, and Cox2 [6]. Many studies have shown that the transgenic overexpression of YAP or mutational inactivation of the upstream regulator of YAP in the liver leads to the dysregulation of organ size and, eventually, hepatocellular carcinoma (HCC) [7–14]. Clinical data also suggest that high YAP expression is associated with poor survival rates in patients with HCC [15,16]. Recent research suggests that high YAP expression in 137 patients with HCC is positively correlated with tumor size, liver cirrhosis, vascular invasion and intrahepatic metastasis [17]. Moreover, some research has revealed that targeted therapeutics against Hippo-YAP signaling might address the challenge of treating advanced-stage HCC because standard chemotherapy is largely ineffective [11,12,18]. These data indicate that

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the Hippo/YAP signaling pathway is critical for HCC progression. However, the exact mechanism underlying YAP dysregulation in the liver remains unclear. A better understanding of the mechanisms regulating the Hippo signaling pathway would contribute to the development of new drugs for the treatment of liver cancer.

Cluster of differentiation 44 (CD44), a principal transmembrane adhesion receptor for hyaluronic acid (HA), belongs to a family of multifunctional transmembrane glycoproteins and is an important regulator of the signal transduction processes that control cell proliferation, survival and differentiation. CD44 is upregulated in a broad range of malignant tumors, and its elevated expression was correlated with poor prognosis in some cancer types [19–22]. Hu et al. reported that standard CD44 (CD44S) expression is correlated with high AFP levels, advanced tumor stage and grade, portal vein tumor thrombus, and early tumor recurrence or metastasis in 128 HCC tissues [23]. However, another study showed that CD44 expression is not significantly associated with tumor differentiation, the AFP levels or disease-free survival in HCC patients [24]. These inconsistent findings suggest that further research must be performed to elucidate the molecular mechanism of CD44 in cancer.

Many membrane receptors, such as integrin receptors and G protein-coupled receptors, have been identified as regulators of the Hippo signaling pathway [25–27]. Previous research has shown that CD44 attenuates activation of the Hippo signaling pathway to promote tumor cell resistance to reactive oxygen species and cytotoxic agent-induced stress [28]. Yu et al. further discovered that CD44 functions as an upstream factor involved in novel crosstalk among the Erk, Akt and Hippo-YAP pathways [29]. Recently, our laboratory found that CD44 could act through RhoA signaling to regulate YAP expression [30]. Although these studies have raised the exciting possibility that CD44 might be an upstream regulator of the Hippo-YAP pathway, the exact relationship between CD44 and this pathway must be clarified, particularly in HCC, and the oncogenic function of CD44 has yet to be fully established.

In this study, we show that CD44S expression is positively correlated with YAP1 expression in HCC tissues, and both proteins are associated with a younger age at diagnosis and a poor pathology grade. Furthermore, we reveal a novel mechanism through which CD44S positively regulates the expression of YAP1 and its target genes via the PI3K/Akt pathway and is regulated by the YAP1/TEAD axis, forming a positive feedback loop. This feedback loop between CD44S and YAP1 might constitute a broadly conserved module that promotes HCC cell proliferation and invasion. Blocking the positive feedback loop involving CD44S and YAP1 might represent a new strategy for the treatment of HCC.

2. Materials and methods

2.1. Tissue samples and study cohort

Forty pairs of tumor samples and matched adjacent nontumor tissues were obtained from the Biobank of West China Hospital of Sichuan University. All the patients signed informed consent forms. This study was approved by the Ethics Committee of West China Hospital of Sichuan University. CD44S and YAP1 expression was detected in all specimens. The tissue specimens were examined separately by two pathologists in a double-blinded manner without prior knowledge of the clinical status of the specimens.

2.2. Immunohistochemistry

Immunohistochemistry (IHC) was performed on all HCC samples using biotin-streptavidin HRP detection systems. Paraffin-embedded tissue sections were collected. After deparaffinization with xylene and dehydration in a graded alcohol series, the tissue sections were subjected to antigen retrieval by microwaving in sodium citrate buffer for 10 min and then inhibiting endogenous peroxidase activity. After nonspecific binding was blocked, the slides were incubated with CD44S antibody (1:100; Abcam, Cambridge, UK,) or YAP1 antibody (1:200; Santa Cruz Biotechnology, CA, USA) in phosphate-buffered saline (PBS) overnight at 4 °C in a humidified container. Biotinylated secondary antibodies (Zhongshan Golden Bridge Biotechnology Co. Ltd., China) were then used according to the manufacturer's recommendations. The sections were incubated with HRP-streptavidin conjugates appropriate for detecting CD44S and YAP1. The brown color indicative of peroxidase activity was developed by incubation with 0.1% 3,3-diaminobenzidine (Zhongshan Golden Bridge Biotechnology Co. Ltd. China) in distilled water for 1–3 min at room temperature. The appropriate positive and negative controls were included in each IHC assay.

2.3. Staining evaluation

An immunoreactivity score system based on the proportion and intensity of positively stained cells was applied. The two scores were calculated as follows: (1) number of positively stained cells: 0, ≤5%; 1, 6%–25%; 2, 26% to 50%; 3, 51% to 75%; and 4, > 75%; and (2) staining intensity: 0, colorless; 1, pale yellow; 2, yellow; and 3, brown. Scores (1) and (2) were multiplied, and the staining grade was classified as absent (score 0), weak (score 1 to 4), moderate (score 5 to 8) or strong (score 9 to 12). All the sections were scored independently by three experienced pathologists. Tumors with absent or weak immunostaining were classified as having low expression, and tumors with moderate or strong immunostaining were classified as having high expression.

2.4. Cell culture and reagents

Human cell lines (HepG2, BEL-7402 and BEL-7404) were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) newborn calf serum (Gibco) in a humidified incubator at 37 °C with 5% CO_2. Four-kilodalton oligosaccharide-HA (O-HA) and LY294002 were obtained from Dalian Meilun Biotech Co. Ltd. (Dalian, China) and Sigma (St Louis, MO, USA), respectively. Verteporfin (VP) was purchased from Novartis (Visudyne; Novartis AG, Basel, Switzerland).

2.5. RNA interference and plasmid transfection

Cells (1×10^5 cells/well) were seeded into six-well plates and transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen/Life Sciences) according to the manufacturer's instructions. After 48 h, the transfected cells were harvested for further analysis. The small interfering RNA (siRNA) sequences used to knock down CD44S and YAP1 expression were designed by and obtained from GenePharma (Shanghai, China). The sequences are listed in Additional File 1: Table S1. The pcDNA3.1 and pcDNA3.1-CD44S vectors were obtained from our laboratory stocks. The pCMV-Flag-YAP-5SA and pCMV-Flag-YAP-S94A expression plasmids were provided by Professor Zhao Bin. Newly constructed plasmids were purified using an EndoFree Plasmid Mini Kit (CWBIO, China) according to the kit instructions.

2.6. Immunoblotting

Both cells and HCC tissues were lysed in RIPA lysis buffer (Bioteke, Beijing, China). Protein concentrations were measured using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA). A Nuclear and Cytoplasmic Extraction Kit (KeyGEN, Jiangsu, China) was used to prepare cytoplasmic and nuclear extracts according to the manufacturer's instructions. The protein extracts were separated in 12% SDS-polyacrylamide gels and transferred electrophoretically onto a PVDF membrane using a Bio-Rad semidry transfer system. The membranes were blocked with 5% milk and incubated with primary antibodies (diluted 1:500–1,000) overnight at 4 °C. After a washing