Research Article

JOURNAL OF HEPATOLOGY

Defeating EpCAM$^+$ liver cancer stem cells by targeting chromatin remodeling enzyme CHD4 in human hepatocellular carcinoma

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Background & Aims: Hepatocellular carcinoma is composed of a subset of cells with enhanced tumorigenicity and chemoresistance that are called cancer stem (or stem-like) cells. We explored the role of chromodomain-helicase-DNA-binding protein 4, which is encoded by the *CHD4* gene and is known to epigenetically control gene regulation and DNA damage responses in EpCAM$^+$ liver cancer stem cells.

Methods: Gene and protein expression profiles were determined by microarray and immunohistochemistry in 245 and 144 hepatocellular carcinoma patients, respectively. The relationship between gene/protein expression and prognosis was examined. The functional role of CHD4 was evaluated in primary hepatocellular carcinoma cells and in cell lines *in vitro* and *in vivo*.

Results: CHD4 was abundantly expressed in EpCAM$^+$ hepatocellular carcinoma with expression of hepatic stem cell markers and poor prognosis in two independent cohorts. In cell lines, *CHD4* knockdown increased chemosensitivity and *CHD4* overexpression induced epirubicin chemoresistance. To inhibit the functions of CHD4 that are mediated through histone deacetylase and poly (ADP-ribose) polymerase, we evaluated the effect of the histone deacetylase inhibitor suberohydroxamic acid and the poly (ADP-ribose) polymerase inhibitor AG-014699. Treatment with either suberohydroxamic acid or AG-014699 reduced the number of EpCAM$^+$ liver cancer stem cells *in vitro*, and suberohydroxamic acid and AG-014699 in combination successfully inhibited tumor growth in a mouse xenograft model.

Conclusions: CHD4 plays a pivotal role in chemoresistance and the maintenance of stemness in liver cancer stem cells and is therefore a good target for the eradication of hepatocellular carcinoma.

Keywords: Chromodomain-helicase-DNA-binding protein 4; Liver cancer stem cells; Histone deacetylase; Poly (ADP-ribose) polymerase; Chemoresistance.

Received 4 December 2014; received in revised form 3 June 2015; accepted 10 June 2015; available online 18 June 2015

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Abbreviations: AFP, alpha-fetoprotein; CHD, chromodomain-helicase-DNA-binding proteins; CSC, cancer stem cell; DSBs, double strand breaks; EpCAM, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HpSC, hepatic stem cell-like; NuRD, nucleosome remodeling and histone deacetylase; PARP, poly (ADP-ribose) polymerase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SALL4, sal-like protein 4; SBHA, suberohydroxamic acid.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death worldwide [1,2]. This is partly due to a lack of effective chemotherapeutic options for patients with advanced-stage disease [3]. Various molecular profiling approaches have been used to identify potential therapeutic targets which are specifically activated in HCC [4–8]. Some studies have indicated the importance of evaluating "stemness" in HCC; it reflects the malignant nature of the tumor and closely correlates with a poor prognosis after surgery [9–12]. Recent evidence has also suggested that HCC may conform to the cancer stem cell (CSCs) hypothesis, which proposes that a subset of cells with stem cell features play a fundamental role in tumor maintenance and chemo/radiation resistance [13]. CSCs, also called tumor-initiating cells or cancer stem-like cells, possess stem cell features in their self-renewal and differentiation capacity, and contribute to the formation of heterogeneous tumor cell populations. In HCC, several stem cell markers, including CD133, CD90, CD13, epithelial cell adhesion molecule (EpCAM), and CD24, have been reported to enrich side populations of CSCs [13–15]. We recently reported that the stem cell markers EpCAM and alpha-fetoprotein (AFP) can be used to classify HCC subtypes with distinct gene expression profiles and patient prognoses [11]. In particular, the EpCAM$^+$ AFP$^+$ HCC subtype shares the gene expression features of cells from hepatic stem cell-like (HpSC)-HCC, and exhibits resistance to the chemotherapeutic reagent 5-fluorouracil [16,17]. However, the underlying molecular mechanisms which are responsible for the chemoresistance of EpCAM$^+$ CSCs remain to be identified.

Using gene expression profiling approaches, we recently identified the activation of transcription factor Sal-like protein 4 (SALL4) in EpCAM$^+$ CSCs from HpSC-HCC [18]. SALL4 is a

transcription factor which plays a fundamental role in the maintenance of embryonic stem cells, possibly through interaction with octamer-binding transcription factor 4, sex determining region Y-box 2, and Nanog [19–24]. It has been reported by three independent groups that SALL4 is a biomarker of HCCs with stem-like gene expression signatures and a poor prognosis [18,25,26]. SALL4 was recently found to directly interact with the epigenetic modulator nucleosome remodeling and histone deacetylase (NuRD) complex [27], thereby altering the histone modifications associated with stemness. Indeed, we have demonstrated that SALL4-positive HCCs have high histone deacetylase (HDAC) activity and are chemosensitive to HDAC inhibitors which reduce SALL4 expression [18].

The NuRD complex is a multi-unit chromatin remodeling complex consisting of chromodomain-helicase-DNA-binding proteins (CHDs), metastasis-associated proteins, and HDACs [28]. Interestingly, recent studies have indicated that CHD4, a DNA-binding protein which complexes with the NuRD complex, plays a role in the DNA damage/repair network and is recruited to DNA-damaged sites in a poly (ADP-ribose) polymerase (PARP)-dependent manner [29–32]. However, the role of CHD4 in the chemoresistance of EpCAM$^+$ CSCs remains to be elucidated. In this study, we investigated the role of CHD4, a NuRD complex protein which regulates HDAC activity and the DNA damage response, in the chemoresistance of liver CSCs. We further evaluated the efficacy of an HDAC inhibitor in combination with a PARP inhibitor in blocking CHD4 function in EpCAM$^+$ HCCs.

Materials and methods

Clinical HCC specimens

For microarray analyses, HCC tissues were obtained from 245 patients who had undergone radical resection from 2002 to 2003 at the Liver Cancer Institute (Fudan University, Shanghai, China). For immunohistochemical analyses, HCC tissues and adjacent non-cancerous liver tissues were obtained from 144 patients who had undergone a hepatectomy from 2002 to 2012 at Kanazawa University Hospital, Japan. The pathological diagnoses were performed as previously described [12]. Of these HCC specimens, 38 were obtained fresh and snap-frozen in liquid nitrogen for RNA analysis. An additional fresh HpSC-HCC sample was also obtained from surgical resection and used immediately to prepare a single-cell suspension. All tissue acquisition and experimental procedures were approved by the Ethics Committee and Institutional Review Board of each institute and conformed to the 1975 Declaration of Helsinki. All patients provided written informed consent.

Cell culture and reagents

The human liver cancer cell lines HuH7, HuH1, Hep3B, HLE, HLF, and SK-Hep-1 were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan) or the American Type Culture Collection (Manassas, VA) and were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary HCC tissue samples were dissected and digested with 1 μg/ml type 4 collagenase solution (Sigma–Aldrich Japan K.K., Tokyo, Japan) at 37 °C for 15 min. Contaminating red blood cells were lysed with ammonium chloride solution (STEMCELL Technologies, Vancouver, Canada) on ice for 10 min. CD45$^+$ leukocytes and annexin V$^+$ apoptotic cells were removed from cell suspensions using an autoMACS-pro cell separator and magnet beads (MiltenyiBiotec K.K., Tokyo, Japan). The HDAC inhibitor suberohydroxamic acid (SBHA) and the PARP inhibitor AG-014699 were obtained from Cayman Chemical (Ann Arbor, MI) and Selleck Chemicals (Houston, TX), respectively. Inhibitor stock solutions were prepared in dimethyl sulfoxide and stored at −20 °C until use. The CHD4 expression plasmid pCMV6-AC-GFP-CHD4 (RG224232) was purchased from Origene Technologies, Inc. (Rockville, MD) and the pcDNA3.1 (V790-20) plasmid, which was used as an empty vector control, was purchased from Invitrogen (Carlsbad, CA). CHD4-specific and control siRNAs were purchased from Dharmacon Research, Inc. (Lafayette, CO); the CHD4#1 and CHD4#2 siRNA sequences were "CCCAGAAGAGGAUUUGUCA" and "GGUUUAAGCUCUUAGAACA", respectively. The siRNA constructs were transfected using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) in accordance with the manufacturer's instructions. The expression of the selected genes was determined in triplicate using a 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Each sample was normalized relative to 18S ribosomal RNA expression. The following Applied Biosystems probes used were: *CHD4*, Hs00172349_m1; *EPCAM*, Hs00158980_m1; *HDAC1*, Hs02621185_sl; *AFP*, Hs00173490_m1; *TERT*, Hs00162669_m1; *BMI1*, Hs00409825_g1; *POU5F1*, Hs03005111_g1; and *18S*, Hs99999901_s1.

Western blotting

Whole cell lysates were prepared using radio-immunoprecipitation assay (RIPA) lysis buffer as previously described [33]. Anti-CHD4 monoclonal (Abcam, Cambridge, UK) and anti-β-actin monoclonal (Sigma–Aldrich Japan K.K.) antibodies were used for protein detection. Immune complexes were visualized using enhanced chemiluminescence detection reagents (Amersham Biosciences Corp., Piscataway, NJ) in accordance with the manufacturer's protocol.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed using an anti-CHD4 monoclonal primary antibody (Abcam) and Envision$^+$ kits (DAKO, Carpinteria, CA) in accordance with the manufacturer's instructions. CHD4 expression was evaluated and categorized as CHD4-high (score, 4–5) or CHD4-low (score, 0–3) based on the CHD4-staining score, which was the sum of the positivity score (0–5%, 0; 5–25%, 1; 25–50%, 2; and >50%, 3) and staining intensity score (weak, 0; moderate, 1; and strong, 2) for an area. Dual-color immunohistochemistry was performed using Vector red (Vector Laboratories Inc., Burlingame, CA) and the anti-EpCAM antibody VU-1D9 (Oncogene Research Products, San Diego, CA). For immunofluorescence, an Alexa Fluor® 488-conjugated anti-mouse immunoglobulin G secondary antibody (Molecular Probes, Carlsbad, CA) was used. Fluorescence microscopy was essentially performed as previously described [34]. Caspase-3 activation was determined by immunofluorescence using a cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology Inc., Danvers, MA).

Cell proliferation assays and fluorescence-activated cell sorting (FACS)

For cell proliferation assays, 2×10^3 cells were seeded in 96-well plates and cell proliferation was evaluated in quadruplicate using the Cell Counting Kit-8 (DOJINDO LABORATORIES, Kumamoto, Japan). For flow cytometry and cell sorting, cells were trypsinized, washed, and resuspended in Hank's balanced salt solution (Lonza, Basel, Switzerland) supplemented with 1% HEPES and 2% FBS. Cells were incubated with the FITC-conjugated anti-EpCAM monoclonal antibody BER-EP4 (DAKO) on ice for 30 min prior to analysis using a FACSCalibur or FACSAriaII (BD Biosciences, San Jose, CA).

Measurement of HDAC and PARP activity

HDAC and PARP activities were measured using the Epigenase HDAC Activity/Inhibition Direct Assay Kit (Epigentek Group Inc., Farmingdale, NY) and HT Universal Colorimetric PARP Assay Kit (Trevigen Inc., Gaithersburg, MD), respectively. Briefly, nuclear proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific KK, Tokyo, Japan) and HDAC and PARP activity was measured in triplicate. Calculations were performed in accordance with the manufacturers' protocols.

Animal studies

Cells (1×10^5 HuH7 or primary HCC cells, or 3×10^6 HLF cells) were resuspended in 200 μl of a 1:1 DMEM: Matrigel (BD Biosciences) mixture and subcutaneously injected into 6-week-old non-obese diabetic/severe combined immunodeficiency mice (NOD/NCrCRl-*Prkdc*^{scid}) which were purchased from Charles River Laboratories, Inc. (Wilmington, MA). For each cell type, 20 mice were inoculated.