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Research Paper

Hydroxylase Activity of ASPH Promotes Hepatocellular Carcinoma Metastasis Through Epithelial-to-Mesenchymal Transition Pathway

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ABSTRACT

Over-expression of aspartyl (asparagynal)-β-hydroxylase (ASPH) contributes to hepatocellular carcinoma (HCC) invasiveness, but the role of ASPH hydroxylase activity in this process remains to be defined. As such, the current study investigated the role of ASPH hydroxylase activity in downstream signalling of HCC tumorgenesis and, specifically, metastasis development. Over-expression of wild-type ASPH, but not a hydroxylase mutant, promoted HCC cell migration in vitro, as well as intrahepatic and distant metastases in vivo. The enhanced migration and epithelial to mesenchymal transition (EMT) activation was notably absent in response to hydroxylase activity blockade. Vimentin, a regulator of EMT, interacted with ASPH and likely mediated the effect of ASPH hydroxylase activity with cell migration. The enhanced hydroxylase activity in tumor tissues predicted worse prognoses of HCC patients. Collectively, the hydroxylase activity of ASPH affected HCC metastasis through interacting with vimentin and regulating EMT. As such, ASPH might be a promising therapeutic target of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and third leading cause of cancer-related mortality worldwide [1]. The overall prognosis of HCC patients remains poor due to its aggressive nature, including an invasive phenotype that is mediated by factors yet to be elucidated [2,3].

Aspartyl (asparaginyl) β-hydroxylase (ASPH) is a member of the α-ketoglutarate-dependent dioxygenase family, which adds hydroxyl groups to β carbons of specific aspartate or asparagine residues in epidermal growth factor (EGF)-like domains [4,5]. In addition to full-length ASPH, the ASPH gene locus also encodes three additional truncated transcripts known as humbug, junctate and junction [6–8]. In particular, humbug transcription is driven by the same promoter of ASPH transcripts and utilizes common exons and open reading frames, whereas chooses an alternative and premature 3′ terminal exon that results in lacking the catalytic domain in the C-terminus of ASPH [6,8,9]. This unusual form of exon sharing between ASPH and humbug may suggest that their functions are highly linked.

ASPH has been reported to be one of the most up-regulated genes in HCC and its over-expression in tumor tissues has been associated with aggressive clinicopathological features and decreased survival [10–12]. However, the exact function of ASPH expressed in tumor tissues has not been clearly defined. Evidence based on isoform-specific methods used to investigate the function of ASPH have been limited. The established prognostic role of ASPH was based on immunostaining using antibodies that were raised from N-terminal peptide of ASPH, which predictably recognized humbug as well [13–15]. Moreover, the conventional gain- or loss-of-function assay of ASPH did not rule out the possible involvement of the non-catalytic domain of humbug being independent of the hydroxylase activity [16–18]. In addition, the functions of ASPH hydroxylase

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activity remain unclear, as structural and biochemical studies have failed to define any functional consequence of hydroxylated factor IX and X, known as the substrates of ASPH [19–21]. Recent study has also demonstrated that humbug itself may exert a potential oncogenic role, as observed in gastric and colon cancers2s [22,23]. Therefore, it remains unclear whether ASPH promotes HCC progression through its hydroxylase activity or non-catalytic domains of humbug. Accordingly, the downstream signalling involved in this process have yet to be determined.

The current study sought to investigate the impact of ASPH hydroxylase activity on HCC invasiveness and metastatic potential. The hydroxylase downstream molecular mechanism and the prognostic impact of the ASPH hydroxylase activity were also defined.

2. Materials and Methods

2.1. Cell Lines, Constructs and Primers

Human embryonic kidney 293 cells, and human HCC cell lines that included Huh-7, SMMC-7721, MHCC-97L and EHBC-512, were maintained as previously described [24]. The ASPH coding sequence was amplified from a cDNA library of EHBC-512. The enzymatic loss ASPH mutant was prepared through site mutagenesis of histidine-679, a reported essential residue for ASPH catalytic activities [19,25], to alanine using an in vitro mutagenesis system (Promega, Madison, WI). The coding sequence of vimentin was synthesized by Shanghai Genepharma (Shanghai, China). The shRNA sequences for silencing ASPH and vimentin were 5′-GCGCAGTGTGAGGATGAT-3′ and 5′-GCTAACTACCAAGACACTATT-3′, respectively. The lentivirus of vimentin, wild-type (WT) or mutant ASPH and the shRNA against human ASPH or vimentin were constructed, packaged and harvested by Shanghai Genepharma. The FLAG-ASPH and HA-vimentin plasmids were constructed through the introduction of ASPH and vimentin coding sequence into pFLAG-CMV (Sigma, St. Louis, MO) and pCMV-HA vectors (Clontech, Kusatsu, Shiga Japan). The primers are listed in Table S1.

2.2. Immunostaining and Immunoblot

These experiments were performed as previously described [24]. The polyclonal antibody of ASPH (FE1) was made in our institution using the synthetic peptide antigen of 12 amino acid residues around the Fe^{2+}-binding domain of ASPH. Anti-ASPH (14116-1-AP) (Proteintech) against N-terminal of ASPH, anti-GFP tag (7G9) (M20004) and anti-actin (M25063)(Abmart, Shanghai, China), anti-myc (sc-40) (Santa Cruz, Dallas, TX), anti-E-cadherin (#610404)(BD Biosciences, San Jose, CA), anti-α-catenin(#2131) and anti-γ-catenin (#2309) (Cell Signalling, Danvers, MA), anti-vimentin(ab8978) (Abcam, Cambridge,UK), anti-β-catenin (bs-1165R), anti-AXIN1 (bs-2439R) and anti-NICD (bs-1335R) (Bioss, Shanghai, China) antibodies were used for western blot or immunostaining. The fluorescent 2nd Alexa Fluor 594 antibody (#A-21145) and Hoechst 33,258 (H-1399) (nuclear staining) was purchased from Molecular Probes (Waltham, MA).

2.3. Asp β-Hydroxylation Assay In Vitro

This assay was modified from previously described methods [19,21]. The first EGF-like domain of human factor IX (China Peptides, Hangzhou, China) was used as the substrate for ASPH. Enzymes were prepared from dialyzed cell lysates of 293 cells transfected with WT-ASPH or H679A. The reaction mixture was incubated in a final volume of 50 μl at 37 °C for 30 min, containing 50 mM PIPES, pH 7.0, 100 mM Fe2+, 20 mM alpha ketoglutarate (α-KG), 0.1 mg/ml BSA, and 100 mM substrate. The α-KG concentration was measured before and after the reaction using the α-KG assay kit (Biovision, Milpitas, CA) according to the manufacturer's instruction. The α-KG consumption reflected hydroxylase activity, which was calculated and calibrated without the substrate. All assays were measured in triplicate and repeated at least three times.

2.4. Cell Growth Curve, Cell Cycle, Cell Migration and Cell-Matrix Adhesion Assay

Cell growth curve, cell cycle and cell migration were assayed as previously described [24]. Cell-matrix adhesion assay was carried out in a 96-well-plate. Briefly, 50 μl of tumor cells with a dilution of 4×10^5/ml were added to each well of the 96-well-plate precoated with Matrigel (BD Bioscience). After incubation at 37 °C for 30 min, unattached cells were washed off and cells adhered to the surface were fixed with 4% paraformaldehyde and then stained with crystal violet. After the plate was washed and dried, the crystal violet was dissolved with 40% acetic acid. The absorbance at 550 nm read by spectrophotometer (Molecular Device, Sunnyvale, CA) was used as an index of cell-matrix adhesion capability. Each sample was assayed and measured in triplicate, and all experiments were repeated at least three times.

2.5. HCC Metastasis Model in Nude Mice

Four-week old male BALB/c nude mice were maintained and cared for according to institutional guidelines. The intrahepatic and distant lung metastasis animal models were established as previously described (2010; [24]). Briefly, in the intrahepatic metastasis model, a total of 1×10^6 Huh-7 cells stably transfected with different constructs were injected into the mice in one left lobe of the liver. Animals were sacrificed 3 months after implantation. Intrahepatic metastases were diagnosed by visible tumor nodules on the opposite liver lobe without cell injection. In the lung metastasis model, a total of 1×10^6 MHCC-97L cells stably transfected with different constructs were injected subcutaneously. Animals were sacrificed 3 months after implantation. Lung metastases were diagnosed through visual inspection and confirmed by histological staining. Livers and lungs were excised. The tissue samples were fixed and embedded in paraffin. Paraffin sections were stained with haematoxylin–eosin (H&E) and vimentin for histological examination.

2.6. Pull-Down Assay, Mass Spectrometry and Co-Immunoprecipitation

FLAG-ASPH and HA-vimentin plasmids were transfected into 293 cells. Crude cell lysate was prepared 72 h after transfection. The protein complex interacting with FLAG-ASPH and HA-vimentin was obtained using the FLAG HA Tandem Affinity Purification Kit (Sigma) according to the manufacturer's instruction. Mass spectrometry analysis of pulled-down immunoprecipitant was performed by the Research Center for Proteome Analysis, Shanghai. Co-immunoprecipitation was used for validating the interacted protein identified by mass spectrometry analysis. The rabbit anti-ASPH antibody FE1 and the mouse anti-vimentin antibodies were used to precipitate their target proteins and related protein complex from crude cell extract of MHCC-97L, respectively.

2.7. Prognostic Significance of ASPH Hydroxylase Activity

A training cohort of patients (n = 213) who underwent liver resection for histologically proven HCC at the Eastern Hepatobiliary Surgery Hospital (EHBH) between 2004 and 2008 and a validation cohort of patients (n = 103) operated at the Mengchao Hepatobiliary Surgery Hospital (MHBH) between 2002 and 2008 were used. Inclusion criteria: (i) had grade 0 or 1 of Eastern Cooperative Oncology Group (ECOG) performance status; (ii) had Child-Pugh class A of liver function; (iii) did not have major hepatic portal