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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

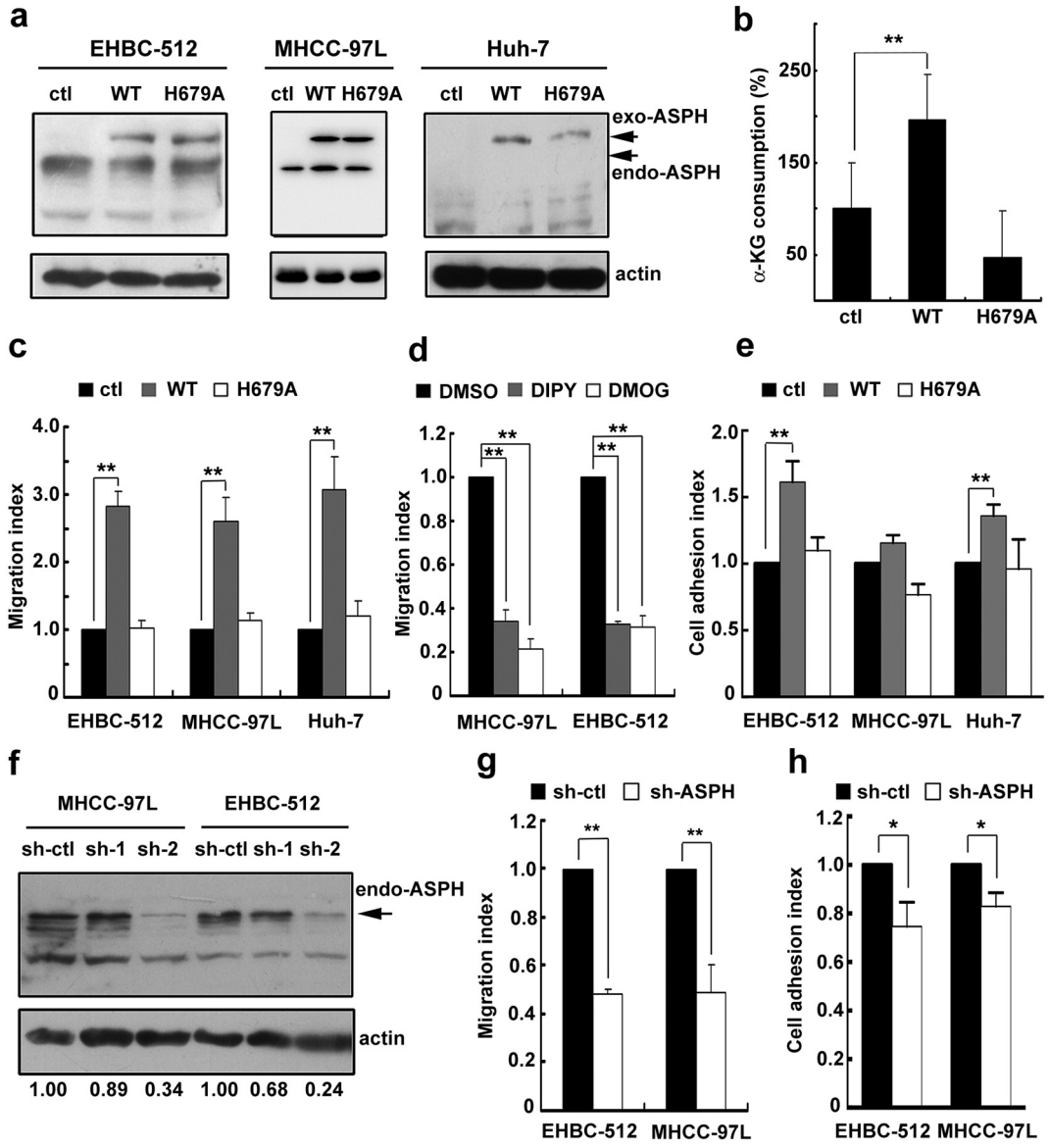


Fig. 1. ASPH hydroxylase activity is required for HCC cell migration and adhesion. (a) Validation of enforced expression of ASPH (wild-type) and its enzymatic mutant (H679A) in MHCC-97L, EHBC-512 and Huh-7 blotted by ASPH antibody specific for C-terminus. *endo*-ASPH, the lower band around 120kD is the endogenously expressed ASPH; *exo*-ASPH, the upper band is the exogenously expressed ASPH that is fused by a GFP tag. (b) The ASPH hydroxylase activity of wild-type and enzymatic mutant of ASPH measured by α-ketoglutarate (α-KG) consumption in in vitro Asp/Asn β-hydroxylation assay in 293 cells transfected with indicated construct. (c) The statistical results of cell migration of MHCC-97L, EHBC-512 and Huh-7 transfected with indicated constructs in the transwell assay. (d) The statistical results of MHCC-97L, EHBC-512 and Huh-7 cell migration upon administration of hydroxylase inhibitor DIPY (1 μM) and DMOG (100 nM). (e) The statistical results of cell adhesion of MHCC-97L, EHBC-512 and Huh-7 transfected with indicated constructs. (f) The silencing of ASPH in MHCC-97L and EHBC-512 cells by lenti-virus mediated shRNA (*sh-1* and *sh-2*) that is testified by immunoblot. The relative quantification of blotting results was shown below. Effective RNAi constructs of *sh-2* were used for later studies. (g) and (h) The statistical results of cell migration or cell adhesion for MHCC-97L and EHBC-512 cells transfected with indicated constructs in the transwell or cell adhesion assay, respectively. All data are shown as average ± SD based on at least three independent experiments after normalization to the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. Abbreviations: ctl or sh-ctl, vector only control group; WT, wild-type of ASPH; H679A, enzymatic mutant of ASPH.

vein tumor thrombus and distant metastasis; and (iv) underwent a R0 liver resection for HCC. Patients who met the criteria were included for prognostic analyses. The clinicopathologic data of all enrolled patients was prospectively collected and maintained in our database. This study was approved by the Institutional Ethics Committees of both centers. Informed consent to use patient data and resected specimens for the purposes of research was obtained from all patients.

The specimen sections were stratified according to the percentages of ASPH positive tumor cells stained with FE1 antibody. Specifically, we defined <10% of FE1 staining positive cells as weak and >10% as intense, suggesting a low and high hydroxylase activity of ASPH, respectively. Histopathological diagnosis and IHC score were carried out independently by three pathologists; disagreements in scoring were adjudicated through discussion among the group.

After surgery, all patients were followed-up regularly once every 2 months within the first 2 years and then once every 3–6 months. The related information of clinical observation is detailed in Tables S6 and S7. At each visit, patients were checked with serum AFP and liver function tests, and abdominal ultrasound. A contrast-enhanced CT or MRI was performed once every 6 months or earlier when clinically indicated. Tumor recurrence was defined by the typical hallmarks of HCC on at least two imaging studies. The HCC recurrence was treated with multimodality options based on general performance, tumor stage, tumor location and cirrhosis. Primary endpoints were overall survival

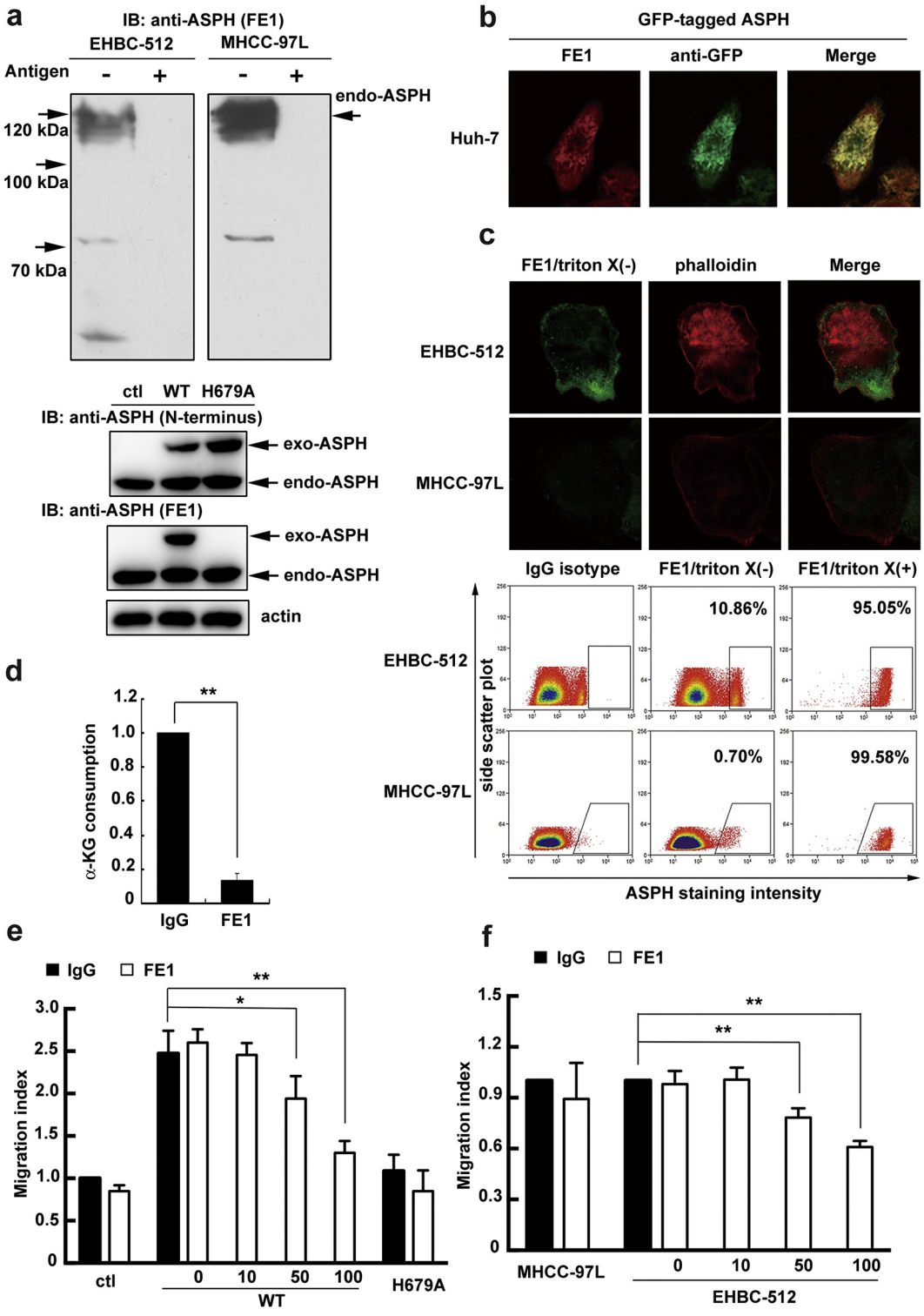


Fig. 2. Blockade of cell migration by a novel antibody FE1 that targets the catalytic domain of ASPH. (a) Validation of the specificity of FE1 by the immunoblot. *Upper*: the peptide competition assay using EHBC-512 and MHCC-97L cell lysate in which FE1 were pre-incubated with the antigen peptide before used in immunoblot. *Bottom*: the specific recognition to wild-type but not enzymatic mutant of ASPH by FE1 using MHCC-97L transfected by indicated constructs. endo-ASPH, the endogenously expressed ASPH; exo-ASPH, the exogenously expressed ASPH that was fused by a GFP tag. (b) Validation of the specificity of FE1 by the immunostaining. Co-localization of positive signal stained by FE1 and anti-GFP antibodies in Huh-7 cells over-expressed with GFP-tagged ASPH (400×). (c) Cell surface expression of ASPH. *Upper*: immunostaining of ASPH by FE1 in impermeable EHBC-512 and MHCC-97L cells without triton X-100 treatment. The cell morphology was characterized by F-actin presence through phalloidin staining. *Bottom*: the presence of cell subsets with membrane or intracellular ASPH expression in EHBC-512 and MHCC-97L cells with or without triton X-100 treatment measured by flow cytometers. (d) The ASPH hydroxylase activity in 293 cells transfected with ASPH upon administrating 100 μg/ml of FE1 antibody or isotype IgG measured by α-ketoglutarate (α-KG) consumption in in vitro Asp/Asn β-hydroxylation assay. (e) and (f) The statistical results of cell migration in Huh-7 cells transfected with indicated constructs, EHBC-512 and MHCC-97L cells that were treated by 100 μg/ml of FE1 antibody or isotype IgG, otherwise the FE1 concentration was designated. All data are shown as average ± SD based on at least three independent experiments after normalization to the control group. *P < 0.05, **P < 0.01 vs. IgG treatment. Abbreviations: ctl, vector only control group; WT, wild type of ASPH; H679A, enzymatic mutant of ASPH.

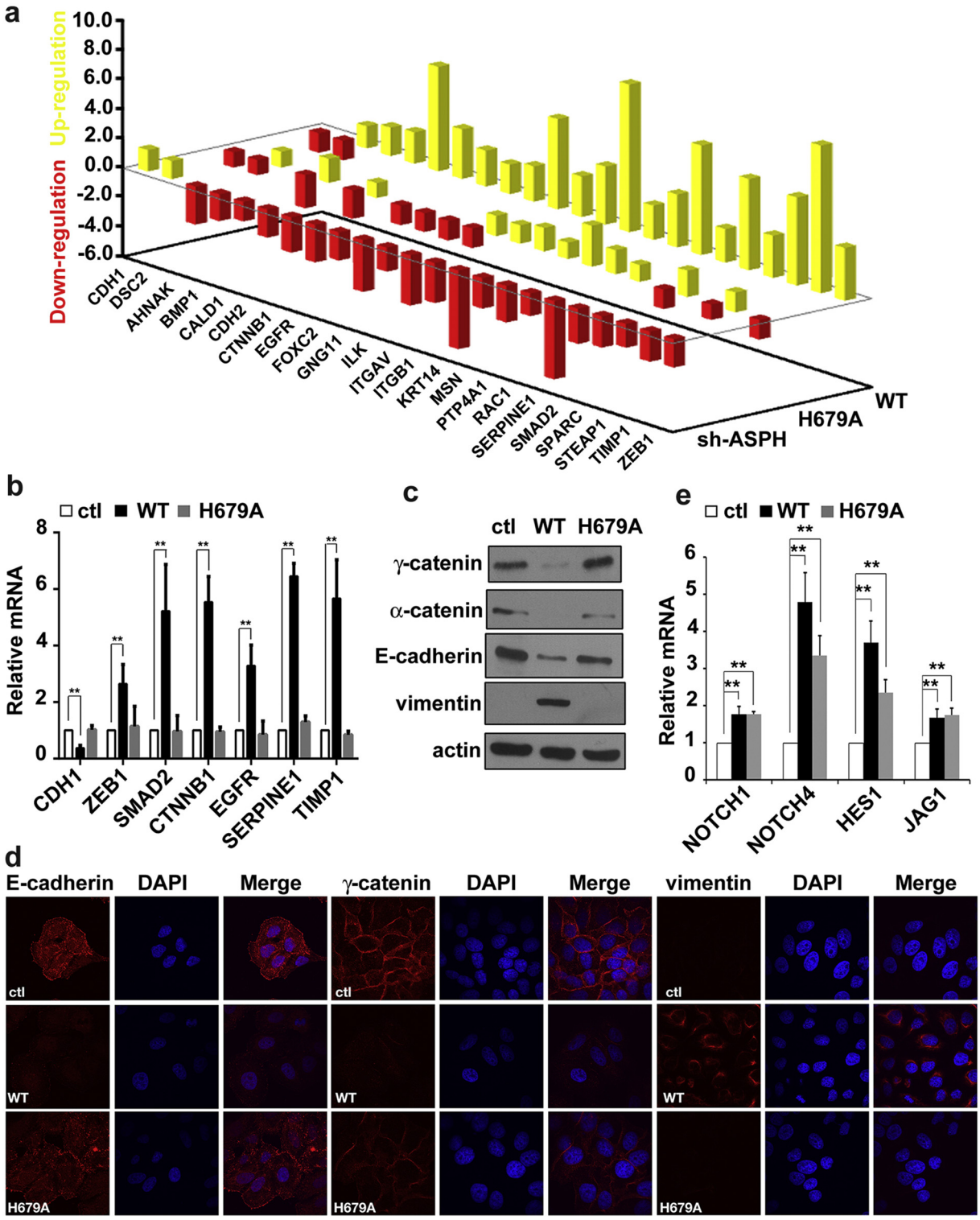


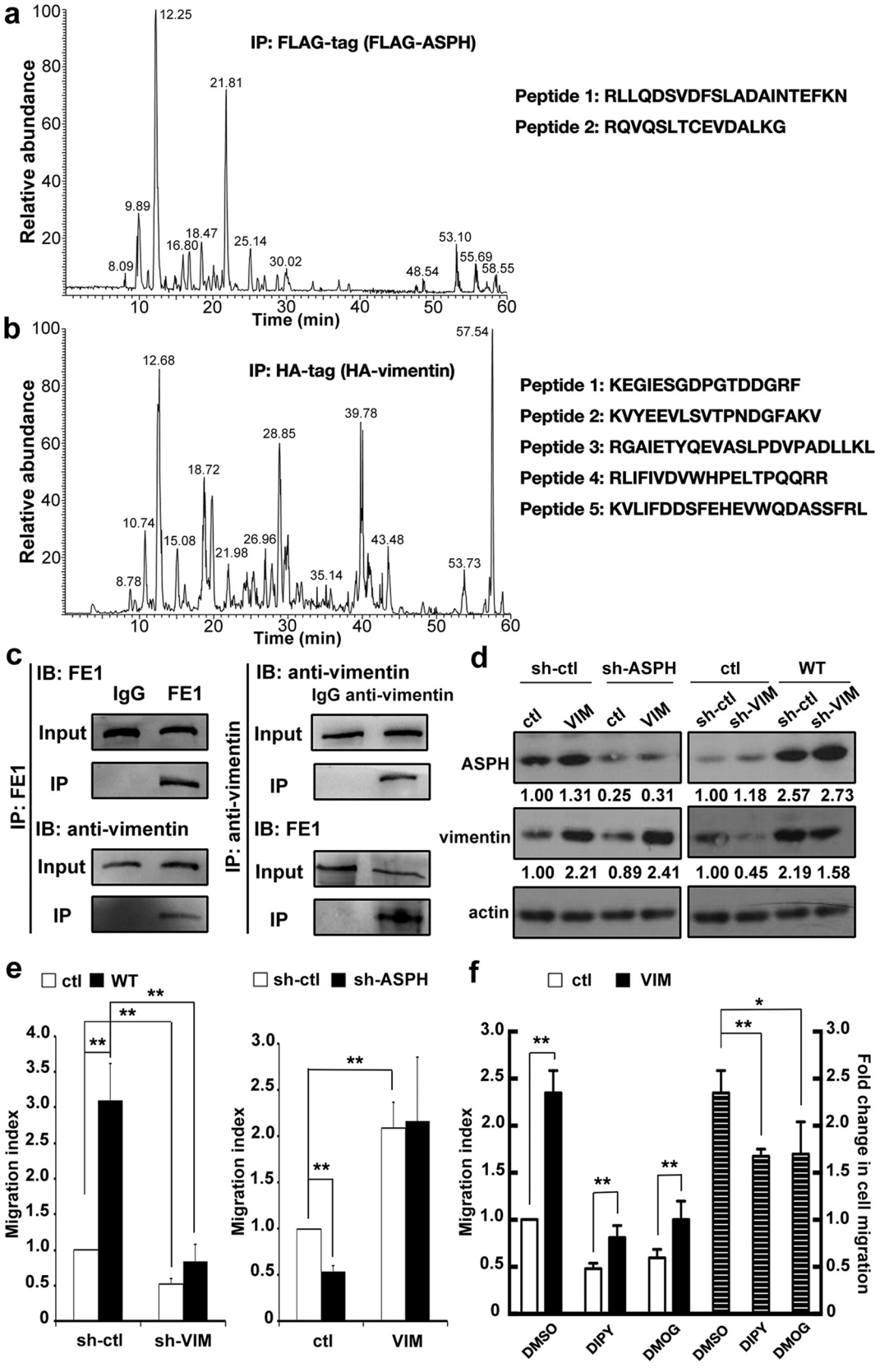
Fig. 3. ASPH hydroxylase activity regulate epithelial-to-mesenchymal transition of HCC cells. (a) The relative up- or down-regulation of EMT biomarkers and regulatory genes in EHBC-512 cells transfected with indicated constructs by the EMT PCR-array normalized by control cells transfected with vector only. (b) The validation of gene expression based on PCR-array results in EHBC-512 cells transfected with indicated constructs. (c) and (d) The immunoblot and immunostaining of EMT biomarker including γ-catenin, α-catenin, E-cadherin and vimentin in Huh-7 cells transfected with indicated constructs. Fluorescent images were taken under 600× magnification. (e) The activation of notch pathway genes in EHBC-512 cells transfected with indicated constructs. Empty vector served as control. *P < 0.05, **P < 0.01 vs. control. All data are shown as average ± SD based on at least three independent experiments after normalization to the control group. *P < 0.05, **P < 0.01 vs. IgG treatment. Abbreviations: ctl, vector only control group; WT, wild type of ASPH; H679A, enzymatic mutant of ASPH.

(OS), defined as the interval between the date of surgery and the date of patient death or last follow-up; other endpoints included time to recurrence (TTR), which was the interval between the date of surgery and the date of diagnosis of HCC recurrence. The follow-up was censored on June 2012.

2.8. Statistical Analysis

Statistical analyses were performed as previously described [12,24]. Statistical analyses were performed using SPSS (version 18.0, Chicago, IL). Continuous variables were expressed as mean ± SD or SEM, or as

indicated. Quantitative values were compared using the Mann-Whitney nonparametric *U* test or Kruskal-Wallis test. Categorical variables were reported as the number of cases and the prevalence, and differences between the groups were compared using the $\chi 2$ test with Yates correction or Fisher exact test as appropriate. The OS and TTR were analyzed using the Kaplan-Meier method and the log-rank test. Independent risk factors were identified using the Cox proportional hazard model. A $p < 0.05$ was considered statistically significant.



3. Results

3.1. Hydroxylase Activity of ASPH is Required for HCC Migration

The WT and enzymatic mutant (H679A) of ASPH were constructed and transfected into human HCC cell lines MHCC-97L, EHBC-512 and Huh-7 (Figs. 1a and S1). In the enzymatic assay for Asp β-hydroxylation, cell lysates from H679A displayed less α-KG consumption than those cells with WT transfection, suggesting a reduced hydroxylase activity of the mutant. In fact, there was up to 76% (148/195) blockade of hydroxylase activity in H679A compared with WT-ASPH (Fig. 1b).

The impact of enhanced ASPH hydroxylase activity on cell growth, cell cycle progression, cell migration and cell adhesion in these transfected HCC cell lines was determined. Over-expression of WT-ASPH, but not H679A, enhanced cell migration in the transwell assay (Figs. 1c and S2a). In contrast, blockade of ASPH activity by 2,2′-dipyridyl (DIPY) and dimethyloxalylglycine (DMOG), two inhibitors of hydroxylase, decreased cell migration (Fig. 1d). In addition, only HCC cells with enforced expression of WT-ASPH demonstrated enhanced cell adhesion (Fig. 1e) compared with cells transfected with control vector or H679A in EHBC-512 and Huh-7 cell lines.

EHBC-512 and MHCC-97H, which had endogenous ASPH expression, were used to selectively silence ASPH (Fig. 1f). Effective depletion of ASPH through shRNA also inhibited HCC cell migration (Figs. 1g and S2b) and cell-matrix adhesion (Fig. 1h). Of note, cell growth and cell cycle profile were unaffected by the change of ASPH expression level (Fig. S3a and b).

3.2. Specific Blockade of ASPH Hydroxylase Inhibits HCC Cell Migration

A polyclonal antibody (FE1) against the Fe-binding His-2 motif at the C-terminal of ASPH, a key region for hydroxylase activity, was prepared. As noted in Fig. 2a *upper*, FE1 specifically recognized endogenous ASPH in EHBC-512 and MHCC-97L, which were sensitive to antigen peptide competition. Unlike other antibodies targeting N-terminal of ASPH (Proteintech, Rosemont, IL), FE1 only recognized the WT-ASPH, but not the enzymatic mutant of ASPH (Fig. 2a lower). Co-immunostaining results demonstrated co-localization of FE1 positive signal and GFP fluorescence that was fused to exogenous ASPH (Fig. 2b).

Given previous reports that ASPH might translocate to the cell membrane in malignant cells [13,16], we performed cell surface ASPH immunostaining in which permeabilizing cells by triton X-100 were omitted. Only EHBC-512 cells showed a clear membrane ASPH presence, whereas MHCC-97L cells did not (Fig. 2c *upper*). The flow cytometer analysis also demonstrated that a certain amount of EHBC-512 cells expressed ASPH on its cell surface, although nearly all EHBC-512 and an MHCC-97L cells intracellularly expressed ASPH (Fig. 2c *lower*).

In addition to its high specificity to ASPH, FE1 also greatly decreased α-KG consumption in the enzymatic assay for Asp β-hydroxylation compared with the IgG isotype control (Fig. 2d), suggesting the antibody was capable of neutralizing the hydroxylase activity of ASPH. The effect of this catalytically inhibitory antibody on migration of tumor cells with membrane ASPH expression was then examined. FE1 displayed a dose-dependent inhibitory effect on cell migration in Huh-7 cells that had over-expression of WT-ASPH (Figs. 2e and S2c). The IC50 of FE1 was around 100 μg/ml, resulting in 80% enzymatic inhibition in the hydroxylase activity assay (Fig. 2d). Of note, FE1 did not block cell migration in Huh-7 cells over-expressed with H679A (Fig. 2e). Likewise, FE1 did inhibit cell migration in EHBC-512 cells that were positive for membrane ASPH in a dose-dependent manner, but was ineffective in MHCC-97L cells with low level of membrane ASPH (Fig. 2f).

3.3. Hydroxylase Activity of ASPH Promotes the Epithelial-to-Mesenchymal Transition

A gene co-expression analysis was performed to search for genes demonstrating a similar expression pattern with ASPH across HCC samples using previously prepared microarray and bioinformatics methodologies [10,26]. The down-regulation of CDH1 (E-cadherin) and RGS2 levels, and up-regulation of TIMP1, ITGB1 and COL1A2 levels were associated with enhanced ASPH levels, implying that the occurrence of epithelial-to-mesenchymal transition (EMT) was connected with ASPH over-expression (Fig. S4).

To better understand the relation between EMT signalling activation and ASPH hydroxylase activity, we examined the change of mRNA expression of EMT biomarkers and regulatory genes through a PCR array using EHBC-512 cells that either had ASPH over-expressed, silenced or mutated compared with control cells, respectively. As noted in Fig. 3a, ASPH over-expression in EHBC-512 cells induced up-regulation of genes that promote EMT, while simultaneously causing down-regulation of genes that inhibit EMT. However, over-expressing H679A did not affect mRNA expression of those EMT-related genes. Moreover, silencing ASPH conferred an opposite effect compared with EMT-related gene expression as did ASPH over-expression. The result of the PCR array was further validated using real-time PCR, which again confirmed that the ASPH activity regulated gene expression profile was associated with EMT (Fig. 3b).

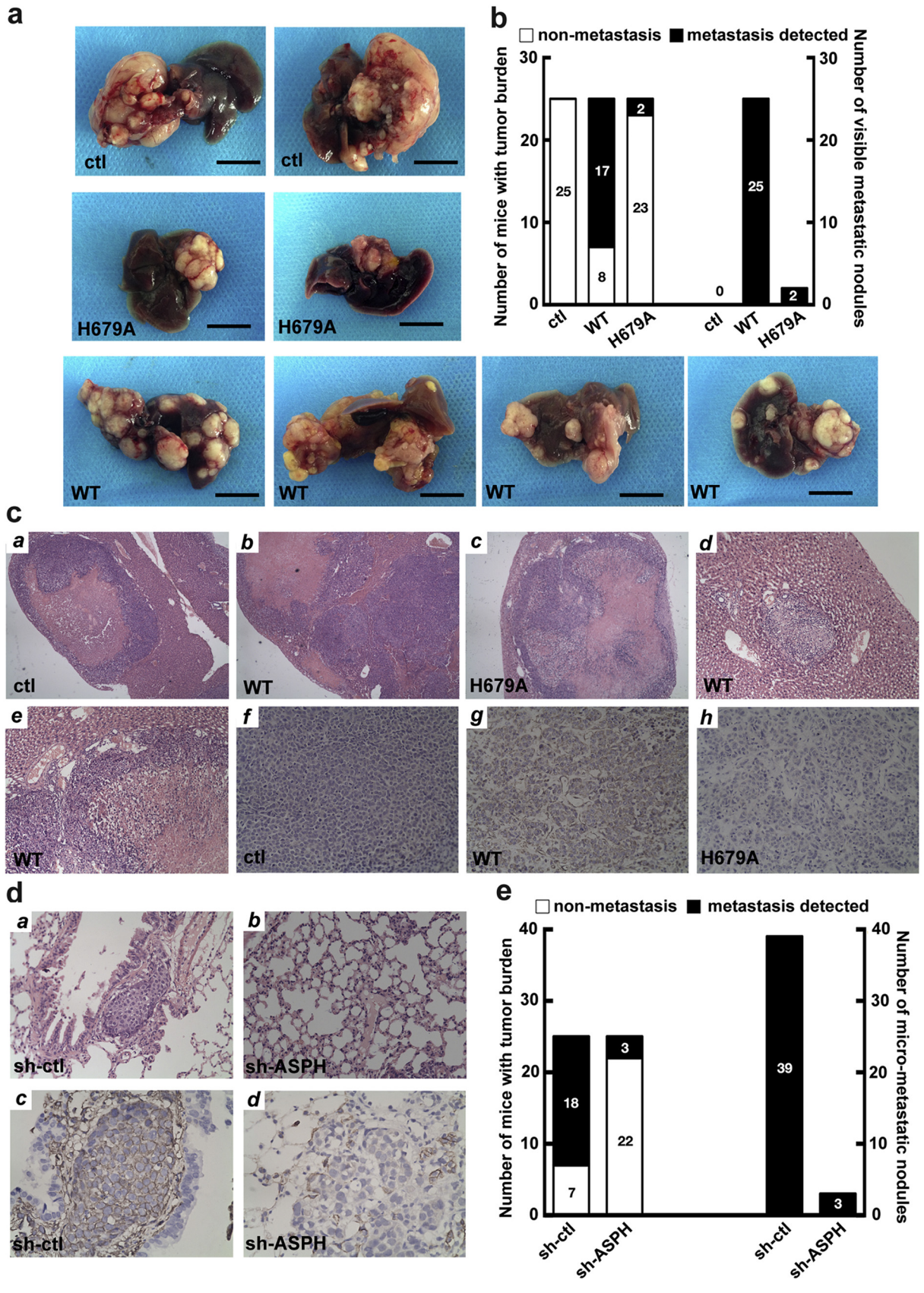
The activation of EMT signalling by enhanced ASPH hydroxylase activity was also verified in Huh-7 cells using immunostaining and immunoblot techniques. These experiments demonstrated that Huh-7 cells over-expressed with WT-ASPH, but not mutant H679A, diminished expression of epithelial cell markers including γ-catenin, α-catenin and E-cadherin, while enhancing expression of vimentin, a mesenchymal marker compared with control constructs (Fig. 3c and d). It has been reported that Wnt-β-catenin pathway plays significant role in EMT [27]. Our data showed that over-expression of WT-ASPH, but not H679A mutant, diminished the RNA and protein levels of AXIN1, a negative regulator of Wnt-β-catenin pathway in Huh-7 cells, while enhanced the protein level of β-catenin (Fig. S5a and b), as well as RNA level of β-catenin (Fig. 3b).

Previous reports had suggested that the notch pathway might be the putative downstream signalling of ASPH [16,28,29]. Consistent with these findings, we similarly noted that ASPH over-expression in EHBC-512 cells induced an increased mRNA level of notch pathway (Fig. 3e). However, over-expressing H679A also enhanced mRNA levels of Notch1, Hes1 and Jag1 compared with the control group (Fig. 3e). In addition, ASPH over-expression in EHBC-512 cells slightly enhanced Notch 1 intracellular domain (NICD) nuclear translocation compared with control and H679A (Fig. S6).

Fig. 4. The role of ASPH-vimentin interaction in promoting HCC cell migration. (a) and (b) Identification of exogenous ASPH-vimentin interaction. *Left*: The base-peak plot of mass spectrometry analysis of protein complex from pull-down assay in 293 cells over-expressed with FLAG-fusion ASPH or HA-fusion vimentin using protein tag antibodies. *Right*: identified peptide sequence belonging to vimentin and ASPH in the protein complex. (c) Validation of endogenous ASPH-vimentin interaction. The immunoblot (IB) of the protein immuno-precipitated (IP) with FE1 and anti-vimentin in MHCC-97 cells. (d) Validation of manipulated vimentin expression in MHCC-97L and Huh-7 cells. *Left*: complementary over-expression of vimentin control and ASPH-silenced MHCC-97L cells. *Right*: complementary silencing vimentin in control and ASPH-over-expressed Huh-7 cells. The relative quantification of blotting results is shown below. (e) The indispensable role of vimentin for ASPH in regulating cell migration. *Left*: functional blockade of cell migration by silencing vimentin in ASPH-over-expressed Huh-7 cells. *Right*: functional rescue cell migration by over-expressing vimentin in ASPH-silenced in MHCC-97L cells. (f) Effect of hydroxylase inhibition to vimentin-dependent cell migration. *Left y axis*: the statistical results of cell migration in vimentin-over-expressed MHCC-97L cells that is treated by DIPY (1 μM) and DMOG (100 nM). *right y axis*: the corresponding increased fold of cell migration by vimentin over-expression in comparison to control group. All data are shown as average ± SD based on at least three independent experiments after normalization to the control group. *$P < 0.05$, **$P < 0.01$ vs. control. Abbreviations: ctl or sh-ctl, vector only control group; WT, wild type of ASPH; VIM: vimentin; sh-ASPH, ASPH silencing; sh-VIM, vimentin silencing.

3.4. ASPH Interacts with Vimentin to Promote HCC Cell Migration

For identifying the direct downstream molecule of ASPH, we performed the pull-down assay to obtain the protein complex interacting with ASPH over-expressed with FLAG tagged-ASPH using 293 cells as a modelling cell mainly due to its high transfection efficiency and low endogenous expression of ASPH. In mass spectrometry, vimentin peptides could be detected in the protein complex precipitated by anti-



Flag antibodies (Fig. 4a). In addition, the presence of ASPH peptides in the protein complex was also observed from the pull-down assay of HA tagged-vimentin (Fig. 4b). The interaction between endogenous ASPH and vimentin was further identified by reciprocal immunoprecipitation and immunoblot by vimentin and ASPH antibodies, respectively, in MHCC-97L cells (Fig. 4c).

For evaluating the mediator effect of vimentin for ASPH, we sequentially over-expressed or silenced ASPH and vimentin in HCC cells based on the cell's established ASPH expression status, showing that over-expression of ASPH enhanced the expression of vimentin and vice versa, and down-regulation of ASPH slightly decreased the expression of vimentin (Fig. 4d). In Fig. 4e, over-expression of vimentin itself greatly promoted HCC cell migration, while knockdown of vimentin exerted an inhibitory effect. In particular, over-expression of vimentin effectively reversed the inhibitory effect of cell migration caused by knockdown of ASPH. Also, tumor cells over-expressed with ASPH failed to display any enhancement of cell migration after vimentin was selectively silenced.

In order to test the relationship between ASPH hydroxylase activity and vimentin function, we analyzed the effect of the hydroxylase inhibitors, DIPY and DMOG, on vimentin-dependent promotion of cell migration. As noted in Fig. 4f, enzymatic blockade of ASPH did not completely eliminate, but indeed weaken the extent of enhancement of cell migration in MHCC-97L cells induced by enforced expression of vimentin, suggesting that ASPH hydroxylase activity might affect vimentin function.

3.5. Hydroxylase Activity of ASPH is Required for HCC Metastasis In Vivo

The metastatic capability of the different HCC cell lines and the number of generated metastatic nodules are summarized in Table S2. In animal models with intrahepatic metastasis, Huh-7 cells that had over-expression of WT-ASPH had a higher probability of intrahepatic metastasis (17/25) and more metastatic nodules (25) in transplanted mice compared with H679A (2/25 and 2, respectively) and control vectors (0/25 and 0, respectively) (Fig. 5a and b). However, there was no obvious difference in the primary tumor size that was formed by Huh-7 cells over-expressing WT-ASPH, H679A, and control vectors on histological examination (Fig. 5a and c); of note, some aggressive pathological features such as microsatellite lesion formation and stromal infiltration were, however, only observed in the WT-ASPH group (Fig. 5c). Consistent with our in vitro findings, only over-expression of WT-ASPH promoted vimentin expression in tumor tissues compared with control and H679A (Fig. 5c). In addition, immunostaining with FE1 and anti-vimentin showed that the intense ASPH staining was well correlated with the intense vimentin staining in HCC tissues from surgical specimens (Fig. S7).

In animal models with lung metastasis, silencing ASPH in MHCC-97L cells decreased the probability of distant metastasis (3/25) and resulted in less lung metastatic nodules (3) compared with the nonsense control group that had high endogenous ASPH expression (18/25 and 39, respectively) (Fig. 5d and e). In particular, lung metastatic lesions formed by MHCC-97L cells demonstrated a clear EMT characteristic in terms of vimentin expression which was significantly lost after ASPH was selectively silenced (Fig. 5d).

3.6. Enhanced ASPH Hydroxylase Activity is Associated With a Poor Prognosis

Using FE1 immunostaining, the tumoral ASPH hydroxylase activity was tested (Table S3). The staining intensity was differentially up-regulated in tumor (intense vs. weak: 122 patients, 57.3% (122/213) vs. 91 patients, 42.7% (91/213) versus non-tumor (80, 37.6% (80/213) vs. 133, 62.4% (133/213), $P = 0.01$) tissues in the training cohort (Figs. 6a and Table S4); these findings were confirmed in the validation cohort.

In the training cohort, there was also a significant association between enhanced ASPH hydroxylase activity and higher serum alpha-fetoprotein (AFP) level, larger tumor size, multiple nodules, microvascular invasion, and late tumor stages according to the Barcelona Clinic Liver Cancer (BCLC) system ($P = 0.038$) and the TNM system (Table S5). Patients with increased ASPH hydroxylase activity had a higher incidence of recurrence at 5-years (78.6% vs. 48.4% for the intense and weak FE1 signalling groups respectively), as well as a lower 5-year OS (35.8% vs. 55.8%) compared with patients who had tumors with low ASPH hydroxylase activity (Fig. 6b). For patients with early stage HCC defined as BCLC stage 0/A, an increased hydroxylase activity also predicted a poor prognosis (5-year recurrence: 61.0% vs. 40.4%; 5-year OS: 42.4% vs. 68.2%, for the intense and weak FE1 signalling groups respectively) (Fig. 6b). Again, these results were validated in the validation cohort (Fig. 6c).

Univariable and multivariable analyses demonstrated that the intensity of FE1 staining was an independent risk factor for both tumor recurrence and OS in the training cohort (hazard ratio: 1.619, 95% confidence interval: 1.109–2.363; 1.987, 1.252–2.129), as well as the validation cohort (1.838, 1.033–3.271; 2.608, 1.121–3.817) (Tables S6 and S7).

4. Discussion

In this study, we defined and characterized the important role that the hydroxylase activity of ASPH plays in HCC metastasis in vitro and in vivo. Particularly, this role has been demonstrated in nude mice with intrahepatic and distant metastases of HCC. Specifically, the interaction between the hydroxylase activity and vimentin regulated EMT in HCC cell lines. In addition, enhanced ASPH hydroxylase activity in HCC tissues was closely associated with aggressive clinicopathological features and a decreased survival outcome.

To date, understanding the biological function of post-translational modification of β-hydroxyasparaginyl/β-hydroxyaspartyl has been very limiteds [6,20]. Although the role of ASPH had been demonstrated in some human malignancies [13,14,30–33], the biological consequences of ASPH hydroxylase activity had remained much less clear. In the current study, we utilized enzymatic mutant analysis and performed single site mutagenesis (H679A) of a highly conserved and essential histidine within the enzymatic domain of ASPH and demonstrated up to a 76% blockade of hydroxylase activity based on an enzymatic assay. In comparing the invasive and metastatic ability of HCC cell lines that were over-expressed with WT or H679A mutant ASPH, we demonstrated that only over-expression of ASPH with intact hydroxylase activity promoted HCC cell migration in vitro, as well as

Fig. 5. ASPH hydroxylase activity promotes HCC formation and metastasis in vivo. (a) Visible liver tumor formation or intrahepatic metastatic nodules in nude mice transplanted with Huh-7 cells over-expressing control vector, wild type and H679A in the liver ($n = 25$ for each group). *Scale bar*, 1 cm. (b) The quantification of intrahepatic metastasis in the mice with liver tumor formation. *Left y axis*: the number of mice with or without visible intrahepatic metastasis in each group as indicated; *right y axis*: the total number of observed metastatic nodules in each group as indicated. (c) Histological or immunohistochemical examination of the liver tumor. *a*, *b*, *c*, liver tumor formed by Huh-7 cells transfected with control, wild type and H679A, respectively, 100× magnification. *d*, *e*, microsatellite tumor and stromal infiltration found in the liver tumor in the group of ASPH, 200× magnification. *f*, *g*, *h*, immunostaining of vimentin in the liver tumors of indicated groups, 400× magnification. (d) Histological or immunohistochemical examination of metastatic nodules detected in the lungs of mice subcutaneously injected with MHCC-97L cells with ASPH silenced or transfected with control vector only ($n = 25$ for each group). *a*, metastatic nodules in the lungs of mice injected with MHCC-97L cells, 200× magnification. *b*, lungs without metastatic lesion in mice injected with control MHCC-97L cells, 200× magnification. *c*, immunostaining of vimentin in the lung metastatic nodules, 400× magnification. *d*, immunostaining of vimentin in the primary subcutaneous tumor, 400× magnification. (e) The counting of lung metastasis in the mice with subcutaneous tumor formation. *Left y axis*: the number of mice with or without detectable metastasis in each group as indicated; *right y axis*: the total number of observed micro-metastatic lesion in each group as indicated.

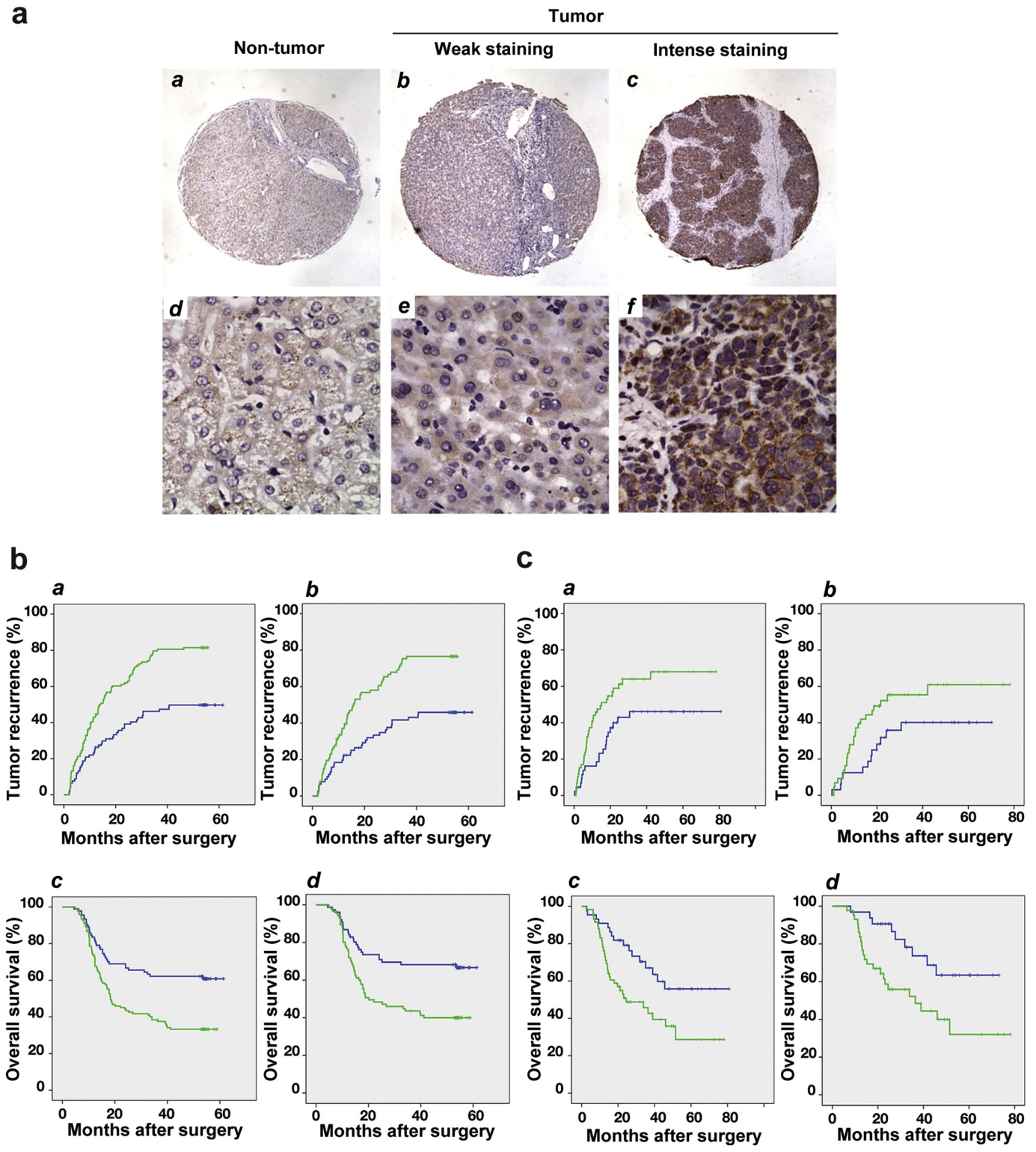


Fig. 6. The ASPH hydroxylase activity is associated with prognosis in HCC patients. (a) Representative images (*upper*, 40× magnifications; *bottom*, 400× magnification) of immunostaining by FE1 antibody in HCC tissue array. *a*, *d*, non-tumor tissues. *b*, *e*, tumor tissues with weak ASPH staining. *c*, *f*, tumor tissue with intense ASPH staining. (b) Kaplan-Meier curve of tumor recurrence and OS after hepatectomy for patients with low (*blue curve*) or high (*green curve*) ASPH hydroxylase activity in the training cohort. *a*, *b*, tumor recurrence in the whole cohort and its BCLC 0/A subgroup. *c*, *d*, OS in the whole cohort and its BCLC 0/A subgroup. (c) Kaplan-Meier curve of tumor recurrence and OS after hepatectomy for patients with low (*blue curve*) or high (*green curve*) ASPH hydroxylase activity in the validation cohort. *a*, *b*, tumor recurrence in the whole cohort and its BCLC 0/A subgroup; *c*, *d*, OS in the whole cohort and its BCLC 0/A subgroup.

tumor intrahepatic and distant lung metastases in vivo. The data demonstrated the essential role of ASPH hydroxylase activity in facilitating invasiveness and metastasis in human malignancies such as HCC in vivo. Consistent with our findings, the small molecule inhibitor MO-I-1100, which reduces ASPH hydroxylase activity, was recently developed and shown to have an anti-tumor effect [10].

Collectively, based on the data, we proposed EMT as a downstream signalling pathway of ASPH hydroxylase activity in HCC. EMT could be

induced by enhanced hydroxylase activity in HCC cells, which is absent from cells transfected with ASPH enzymatic mutant. Furthermore, vimentin was identified as a new interaction partner of ASPH, providing a mechanistic connection between ASPH and EMT. Over-expression of vimentin is associated with HCC development and metastasis [34]. To this point, data from the current study demonstrated that vimentin was critical for cell migration promoted by ASPH hydroxylase activity in the functional complementary assays. Although the direct hydroxylation of vimentin by ASPH needs to be confirmed, results from the current study demonstrated that the enhancement of cell migration through over-expressing vimentin was sensitive to hydroxylase inhibitors, suggesting the importance of ASPH hydroxylase activity in vimentin signalling.

Previous studies have suggested that HIF-1α-ASPH-Notch axis was involved in cell motility regulation [29]. Our data also confirmed that Notch pathway activation, in terms of enhanced mRNA level of Hes1 and Notch1 and nuclear translocation of Notch1, was in response to the enhanced expression of ASPH. Nevertheless, we cannot exclude the potential involvement of HIF-1α as an upstream regulator of ASPH in HCCs.

In earlier clinical studies of ASPH, its expression was mainly assessed by immunostaining using antibodies, which had a high probability of cross-reaction with the non-catalytic humbug given that these antibodies had a higher affinity to humbug rather than ASPH given its C-terminus with hydroxylase activity [12–14,23,32,33]. As such, we made an antibody (FE1) specific for the essential motif within the catalytic domain of ASPH. This antibody had extremely high specificity for ASPH with intact hydroxylase activity, as demonstrated by its not recognizing the H679A mutant. By using FE1 to assess data from a multicentric database, we were able to validate that an increased ASPH hydroxylase activity in HCC tissue contributed to a markedly worse long-term prognosis following curative resection for HCC.

In addition to the lack of cross-reaction with humbug, the FE1 antibody displayed some other distinct advantages. First, the FE1 antibody directly recognized ASPH in the plasma membrane without permeabilizing cells. Second, the antibody could neutralize ASPH hydroxylase activity, which likely resulted from its specific binding to the central motif of the catalytic domain of ASPH [19,25]. Third, FE1 inhibited cell migration through targeting membrane ASPH. The therapeutic potential of ASPH, especially the surface expressed isoform, has been recently confirmed in several studies [10,35–39]. This is not surprising as most predicted ASPH substrates are extracellular matrix (ECM) proteins [40,41] and membrane ASPH sheds its catalytic domain outside of the membrane, facilitating the hydroxylation of ECM by ASPH. Although vimentin is an intracellular protein, it is actually connected to a complicated ECM network through a physical interaction [42–44]. The interaction between ASPH and vimentin provided a possibility that ECM might relay the inhibitory signal upon FE1 binding to surface ASPH to regulate the vimentin function.

One limitation of the current study was that we failed to identify the hydroxylated residues mainly due to technical limitation in detecting β-hydroxyasparaginyl/β-hydroxyaspartyl on the protein. Further study is required to address this shortcoming.

In summary, data from the current study demonstrated the crucial role of ASPH hydroxylase activity in HCC invasiveness and metastatic potential. ASPH hydroxylase activity regulates EMT pathway likely through interacting with vimentin.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Author Contributions

F.S., Y.W., Q.Z. and Y.H. designed the research. Q.Z., Y.H., K.W., X.L., J.L. and B.J. performed the majority of the experiments. Y.X., X.W., J.L., A.H. and D.W. provided clinical specimens and performed clinical observation. H.X., H.W. and W.Y.L. assisted with study design and analyzed data. Q.Z., Y.H., H.W., T.M.P., Y.W. and F.S. conceived this study, wrote and revised the manuscript.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.05.004.

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