Published in final edited form as:

J Hepatol. 2016 November ; 65(5): 888–898. doi:10.1016/j.jhep.2016.05.022.

EpCAM-regulated intramembrane proteolysis induces a cancer stem cell-like gene signature in hepatitis B virus-infected hepatocytes

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Abstract

Background & Aims—Hepatocytes in which the hepatitis B virus (HBV) is replicating exhibit loss of the chromatin modifying polycomb repressive complex 2 (PRC2), resulting in re-expression of specific, cellular PRC2-repressed genes. Epithelial cell adhesion molecule (*EpCAM*) is a PRC2-repressed gene, normally expressed in hepatic progenitors, but re-expressed in hepatic cancer stem cells (hCSCs). Herein, we investigated the functional significance of EpCAM re-expression in HBV-mediated hepatocarcinogenesis.

Methods—Employing molecular approaches (transfections, fluorescence-activated cell sorting, immunoblotting, qRT-PCR), we investigated the role of EpCAM-regulated intramembrane proteolysis (RIP) in HBV replicating cells *in vitro*, and in liver tumors from HBV X/c-myc mice and chronically HBV infected patients.

Results—EpCAM undergoes RIP in HBV replicating cells, activating canonical Wnt signaling. Transfection of Wnt-responsive plasmid expressing green fluorescent protein (GFP) identified a GFP⁺ population of HBV replicating cells. These GFP⁺/Wnt⁺ cells exhibited cisplatin- and sorafenib-resistant growth resembling hCSCs, and increased expression of pluripotency genes

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

OA conceived and supervised the project. OA, SM and PM designed the study and wrote the manuscript. SM, HZ, and AD performed the experiments, collected the data and analyzed the results. PP performed the bioinformatic analyses of human PCR array data. NF, LL, BB and PM provided, annotated and analyzed the human patient samples. PM generated the clinico-pathological patient data. PM, NF, LL generated the beta-catenin sequencing analysis. All authors have read and approved the final manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2016.05.022>.

NANOG, *OCT4*, *SOX2*, and hCSC markers *BAMBI*, *CD44* and *CD133*. These genes are referred as EpCAM RIP and Wnt-induced hCSC-like gene signature. Interestingly, this gene signature is also overexpressed in liver tumors of X/c-myc bitransgenic mice. Clinically, a group of HBV-associated hepatocellular carcinomas was identified, exhibiting elevated expression of the hCSC-like gene signature and associated with reduced overall survival post-surgical resection.

Conclusions—The hCSC-like gene signature offers promise as prognostic tool for classifying subtypes of HBV-induced HCCs. Since EpCAM RIP and Wnt signaling drive expression of this hCSC-like signature, inhibition of these pathways can be explored as therapeutic strategy for this subtype of HBV-associated HCCs.

Lay summary—In this study, we provide evidence for a molecular mechanism by which chronic infection by the hepatitis B virus results in the development of poor prognosis liver cancer. Based on this mechanism our results suggest possible therapeutic interventions.

Keywords

Hepatitis B virus; Hepatocellular carcinoma; EpCAM-regulated intramembrane proteolysis; SUZ12/polycomb repressive complex 2; Pluripotency genes (*NANOG*, *OCT4* and *SOX2*); *CD44*; *CD133*; *BAMBI*; Wnt signaling

Introduction

Chronic hepatitis B virus (HBV) infection is a major factor in the pathogenesis of hepatocellular carcinoma (HCC) [1]. Currently, liver cancer has the most rapidly growing mortality rate in the United States, relative to other cancers, and is the 5th most common cancer world-wide [2]. Despite the HBV vaccine, the World Health Organization estimates 250 million people globally are chronically infected with HBV. Moreover, the HBV vaccine is not always protective; children born of infected mothers become chronically infected. Current treatments include antiviral nucleoside analogs, efficient in suppressing HBV replication, but having no impact on persistence of the viral mini-chromosome within the host hepatocyte, or production of the HBV oncoprotein (HBx) by the integrated viral DNA [3,4]. In advanced stage HCC, sorafenib therapy provides survival improvement, delaying tumor progression [5,6]. However, human liver tumor cells exhibiting mesenchymal features and expressing cluster of differentiation (CD)44, a hepatic cancer stem cell (hCSC) marker [7], are resistant to sorafenib-induced cell death *in vitro* [8]. Thus, new and effective mechanism-based therapies are needed to inhibit deleterious effects of HBx protein on cell homeostasis and to prevent formation of hCSCs, as well as new prognostic tools for precise classification of HCC.

hCSCs detected in liver cancer of various etiologies are characterized by expression of cell surface markers Epithelial cell adhesion molecule (EpCAM) [9], *CD133* [10], *CD90* [11], *CD44* [12], *CACNA2D1* [13] among others [14,15]. However, the molecular mechanism generating formation of hCSCs is not yet understood. Lineage tracing in mice showed that following liver injury, hepatocytes reprogram to a distinct cell population resembling hepatic progenitors [16]. Human hepatocytes also have the same capacity [16]. Since HBV infects differentiated hepatocytes [17], HBV infection must reprogram differentiated hepatocytes to

hepatic-like progenitors during HCC pathogenesis, enabling expression of hCSC markers by a mechanism not yet understood.

Regarding this mechanism, liver tumors from animals modeling HBV-mediated hepatocarcinogenesis and also HBV replicating cells exhibit loss-of-function of the chromatin modifying polycomb repressive complex 2 PRC2 complex [18,19]. PRC2, comprised of three core subunits, SUZ12, EED and methyltransferase EZH2, silences transcription by trimethylation of H3 on K27 [20], thereby regulating cell fate outcomes during lineage commitment [21]. HBx, a co-factor in HBV-mediated hepatocarcinogenesis [22,23], induces proteasomal degradation of SUZ12 [24]. Loss of PRC2 function in HBV replicating cells [19] allows re-expression of specific PRC2 target genes, including *EpCAM* and *BAMBI*, normally expressed in hepatic progenitors [25,26]. In this study we investigated the functional significance of EpCAM re-expression in HBV replicating cells and its contribution to HCC pathogenesis.

EpCAM undergoes regulated intramembrane proteolysis (RIP) in human colon cell lines, generating the intracellular C-terminal domain (EpICD). In turn, the EpICD/ β -catenin complex translocates to the nucleus leading to activation of canonical Wnt signaling [27]. Wnt signaling contributes to maintenance of pluripotency [28], and reprogramming of somatic cells to pluripotency [29]. Furthermore, loss of PRC2 function does not affect maintenance of pluripotency in embryonic stem cells [30,31]. Here we show that a subpopulation of HBV replicating cells exhibiting reduced SUZ12 protein levels also exhibits activation of Wnt signaling via EpCAM RIP, resistance to cisplatin and sorafenib, elevated expression of hCSCs markers (*CD44*, *CD133* and *BAMBI*), and pluripotency genes (*NANOG*, *OCT4* and *SOX2*). We refer to these upregulated genes as EpCAM RIP and Wnt-induced hCSC-like gene signature. Clinically, HBV-associated HCCs form distinct subgroups based on expression of this gene signature. Notably, HBV-associated liver tumors that express the hCSC-like gene signature are associated with poor prognosis and reduced overall survival after surgical resection. Since in HBV replicating cells and X/c-myc liver tumors [22], expression of the hCSC-like gene signature is mediated by EpCAM RIP and Wnt pathway activation, inhibition of these pathways could serve as therapeutic strategy for this subtype of HBV-associated HCCs.

Materials and methods

Human liver tissues

Tumor (T) and matched non-tumorous liver parenchyma (NT) (at least 2 cm distant of the tumor burden) were collected from frozen, surgically resected HCCs (French Resource Biological Centre). Clinico-pathological data were obtained from 32 pairs, and survival data from 26 pairs. Eight frozen surgically resected normal liver tissues (N) were from patients with liver resection for colorectal adenocarcinoma metastasis (Centre Léon Bérard Resource Biological Centre, ministerial agreements #AC-2013-1871 and DC-2013-1870). Signed informed patient consent was obtained before surgery. Histological analysis confirmed diagnosis and characteristics of HCCs (T samples), fibrosis stage (NT samples) using the French METAVIR cooperative study group criteria, and absence of microscopic tumor invasion (T and NT samples).

Cell culture

Tetracycline-regulated HBx-expressing cell lines 4pX-1, 4pX-1-*SUZ12*^{KD}, 4pX-1-*p53*^{KD}, and 4pX-1^{GIPZ} (vector control cell line for 4pX-1-*SUZ12*^{KD} and 4pX-1-*p53*^{KD}) were grown as described [18]. HBx expression was induced by tetracycline removal for 18 h, confirmed by PCR at regular intervals of less than 6 months. The HepAD38 cell line obtained from Dr. C. Seeger, Fox Chase Cancer Center, Philadelphia, PA, was cultured as described [32]. Viral replication was induced by tetracycline removal, and expression of HBV pregenomic RNA in HepAD38 cells was tested by PCR [32]. HepG2-NTCP cell line, obtained from Dr. Stefan Urban, Heidelberg Germany, in 2015, was infected with 100 HBV virus genome equivalents (vge), as described [33]. All cell lines used in the study were routinely tested for mycoplasma, and retention of described properties and growth characteristics.

Flow cytometry, MTS assays, immunoblots/antibodies, RNA isolation, cDNA synthesis, qRT-PCR and primers, transfections and luciferase assays

Detailed protocols are provided in Supplementary materials and methods, and Supplementary Tables 1 and 2.

PCR array and data analysis

Custom Taqman PCR arrays were designed containing human-specific primers for 23 genes, constructed by Life Technologies. RNA was isolated from frozen liver samples using RNAqueous Micro kit (Life Technologies) and quantified using nanodrop spectrophotometer. cDNA was synthesized using Superscript VILO cDNA synthesis kit (Life Technologies). cDNA (10 ng) was pre-amplified using Taqman PreAmp master mix (Life Technologies) and Taqman Custom PreAmp pool (Life Technologies). Real time PCR was performed in ViiA7 Real Time PCR system (Life Technologies) using pre-amplified cDNA following manufacture's protocol. Data was processed with custom R scripts. Gene expression was normalized relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and gene-specific z-scores were calculated relative to a pool of normal liver samples. Samples were manually grouped according to their expression of alpha fetoprotein (*AFP*), *EpCAM*, *BAMBI*, *CD133*, *CD44*, *OCT4*, *SOX2* and *NANOG* and visualized as a heatmap.

Statistical analysis

Statistical analyses were performed using unpaired t test. Differences were considered significant when $p < 0.05$. MedCalc software (Version 12.7.1.0) was used for statistical analysis of clinico-pathological patient data. Correlations were calculated by the Spearman correlation test. Log-rank test and Kaplan-Meier method were used to assess survivals. Tests were considered significant when $p < 0.05$.

Results

EpCAM undergoes regulated intramembrane proteolysis (RIP) in HBV replicating cells

Deregulation of Wnt signaling by multiple mechanisms, plays an important role in hepatocarcinogenesis [34]. EpCAM is overexpressed in hCSCs [11] and is induced by loss

of PRC2 function in HBV replicating cells [19]. Since EpCAM RIP activates Wnt signaling [27], we examined whether EpCAM RIP occurs in the presence of HBV replication. The HepAD38 cell line, derived from HepG2 human liver cancer cell line, is a cellular model that supports HBV replication [32]. HepAD38 cells contain an integrated copy of the HBV genome under control of the Tet-off promoter, and HBV replication is initiated by tetracycline removal [32]. In the presence of HBV replication, we quantified by qRT-PCR a small increase in *EpCAM* mRNA levels (Fig. 1A), while flow cytometry showed EpCAM was expressed in nearly all cells (Supplementary Fig. 1A). Interestingly, immunoblots of EpCAM as a function of HBV replication from day 0 to day 20, showed a progressive reduction in EpCAM protein levels, while levels of EpCAM C-terminal fragment (CTF) were increased (Fig. 1B). Like-wise, the level of HBV core antigen (HBc), a marker of HBV replication, was also increased (Fig. 1B). Since EpCAM undergoes RIP by membrane-associated γ -secretase [27], we examined the effect of γ -secretase inhibitor DAPT [27] on EpCAM protein levels in HBV replicating cells. Indeed, treatment with DAPT restored EpCAM protein levels, indicating HBV replication promotes EpCAM RIP (Fig. 1B and C).

To further confirm the observations obtained with HBV replicating HepAD38 cells, we employed the recently developed HBV infection model of the HepG2-NTCP cell line [33]. HepG2-NTCP cells express sodium taurocholate co-transporting peptide (NTCP) that specifically binds HBV preS1 protein and can be directly infected by purified HBV virus. Quantification of viral DNA, cccDNA and HBe antigen were used to detect HBV infection in HepG2-NTCP cells (Supplementary Fig. 1B). HBV infected HepG2-NTCP cells showed a small increase in *EpCAM* mRNA levels. Interestingly, the level of full length EpCAM protein was reduced at day 4 post-infection, while DAPT treatment suppressed this reduction (Fig. 1D), indicating that EpCAM undergoes RIP in HBV infected HepG2-NTCP cells.

HBV replicating cells activate Wnt signaling via EpCAM RIP

EpCAM RIP activates canonical Wnt signaling via nuclear translocation of β -catenin by interaction with intracellular domain EpICD [27]. To determine whether EpCAM RIP observed during HBV replication, activates Wnt signaling, we employed Wnt-responsive TOPflash reporter (Fig. 2A) and immunofluorescence microscopy for β -catenin (Fig. 2B). HBV replicating HepAD38 cells showed enhanced luciferase expression from Wnt-responsive TOPflash reporter, while transfection of two different EpCAM siR-NAs decreased luciferase expression (Fig. 2A). Employing immunofluorescence microscopy, we observed β -catenin membrane immunostaining on day 0 of HBV replication. Interestingly, nuclear β -catenin was observed in some HBV replicating cells (day 10), indicative of Wnt pathway activation (Fig. 2B). Furthermore, inhibition of EpCAM RIP by addition of γ -secretase inhibitor DAPT (29), or knockdown of *EpCAM* by siRNA transfection in HBV replicating cells, increased membrane-associated β -catenin (Fig. 2B). In addition, activation of Wnt signaling was also observed upon HBV infection of HepG2-NTCP cells, based on luciferase activity from transfected TOPflash reporter, while treatment with DAPT abolished induction of luciferase activity (Fig. 2C). To confirm that Wnt signaling is activated in HBV infected HepG2-NTCP cells, we carried immunofluorescence microscopy for HBV S antigen (HBs) and β -catenin. We observed reduced membrane immunostaining of β -catenin

in HBV infected cells immunostained for HBs (Supplementary Fig. 1C). We conclude that EpCAM RIP activates Wnt signaling in HBV infected cells.

EpCAM RIP, Wnt activation, and expression of pluripotency genes in a SUZ12 knockdown cellular model

The *EpCAM* gene is epigenetically silenced by the PRC2 complex in ESCs [35], as well as in hepatocytes [19]. HBV protein X (HBx), essential for HBV replication [36], promotes proteasomal degradation of *SUZ12*, resulting in loss of PRC2 function [19,24]. To understand how HBV infection contributes to EpCAM-mediated Wnt signaling, we employed the *SUZ12* knockdown 4pX-1-*SUZ12*^{KD} cell line [18]. This cell line was constructed in 4pX-1 cells [37] derived from immortalized mouse hepatocyte AML12 cell line [38], and expresses HBx via the Tet-off system [37]. Expression of EpCAM was increased in *SUZ12* knockdown cells in comparison to control 4pX-1^{GIPZ} cells (Fig. 3A). A most pronounced EpCAM induction was quantified by comparing basal expression between 4pX-1^{GIPZ} and 4pX-1-*SUZ12*^{KD} cells. Specifically, a 2.3-fold increase was quantified by immunoblots in absence of HBx between 4pX-1^{GIPZ} and 4pX-1-*SUZ12*^{KD} cells, and a 5-fold increase by qRT-PCR (Fig. 3A). These results indicate that HBx upregulates EpCAM expression via loss of PRC2-mediated EpCAM silencing, mediated by proteasomal degradation of *SUZ12*, as we have shown recently [24].

Next, we investigated whether Wnt signaling was also activated in 4pX-1-*SUZ12*^{KD} cells via EpCAM RIP. Wnt-responsive luciferase vector TOPflash was transfected in 4pX-1^{GIPZ} cells, 4pX-1-*SUZ12*^{KD} and 4pX-1-*p53*^{KD} cells, a *p53* knockdown cell line. Notably, loss of *p53* as often occurs in HBV-mediated HCCs [39], is linked to induction of canonical Wnt signaling [40] and EpCAM induction (Supplementary Fig. 2). In comparison to 4pX-1^{GIPZ} cells, knockdown of *SUZ12* and *p53* enhanced Wnt signaling by 2- and 4-fold, respectively (Fig. 3B). Transfection of *EpCAM* siRNA significantly reduced Wnt signaling (Fig. 3B), supporting EpCAM RIP activates Wnt signaling in *SUZ12* and *p53* knockdown cells.

Canonical Wnt signaling is required for expression of pluripotency genes [28]. Since Wnt signaling is activated in *SUZ12* and *p53* knockdown cells (Fig. 3B), we examined expression of pluripotency genes. Enhanced expression of *NANOG*, *OCT4*, and *SOX2* was detected both by immunoblots (Fig. 3C) and qRT-PCR (Fig. 3D) in 4pX-1-*SUZ12*^{KD} and 4pX-1-*p53*^{KD} cells. To confirm that induction of pluripotency genes is mediated by Wnt signaling, we transfected 4pX-1-*p53*^{KD} cells with a plasmid expressing green fluorescent protein (GFP) from Wnt-responsive TCF/LEF promoter (TOPGFP plasmid). Cells with activated Wnt signaling expressed GFP and were isolated by fluorescence-activated cell sorting (FACS). Compared to GFP⁻ cells, GFP⁺ cells displayed increased expression of *NANOG*, *OCT4*, and *SOX2* (Fig. 3E).

Induction of pluripotency genes in a subpopulation of HBV replicating cells

Similar analysis employing RNA isolated from HBV replicating HepAD38 cells lacked induction of pluripotency genes (Supplementary Fig. 3), despite activation of Wnt signaling (Fig. 2). Since nuclear localization of β -catenin was observed in only some of HBV replicating cells (Fig. 2B), we reasoned a subpopulation of HBV replicating cells were

Wnt⁺. Accordingly, we transfected in HBV replicating HepAD38 and HBV infected HepG2-NTCP cells (data not shown), the TOPGFP plasmid, which also expresses mCherry constitutively. Employing flow cytometry, we quantified increased percentage of GFP⁺ cells in the presence of HBV replication (Fig. 4A-B). Furthermore, treatment with LiCl, a Wnt activator, showed significant increase in GFP⁺ cells confirming that they exhibit Wnt activation (Fig. 4B). By contrast, transfection of *EpCAM* siRNA in HBV replicating cells reduced GFP⁺ cells to basal level (Fig. 4B). Moreover, GFP⁺ cells showed significant increase in *Axin2* expression, a target of Wnt signaling, compared to GFP⁻ cells, demonstrating GFP⁺ cells show significantly higher Wnt activation than GFP⁻ cells (Fig. 4C).

Next, by FACS we isolated the GFP⁺ cells from transfected HBV replicating HepAD38 cells. RNA isolated from sorted GFP⁺ cells was used to quantify expression of pluripotency and hepatic progenitor genes. GFP⁺ cells exhibited increased mRNA levels of hepatic progenitor genes *BAMBI*, *CD44* and *CD133*, as well as pluripotency genes *OCT4*, *SOX2* and *NANOG*, compared to GFP⁻ transfected cells (Fig. 4C). Furthermore, SUZ12 immunoblots demonstrated, both in HBV infected HepG2-NTCP cells as well as sorted GFP⁺ HBV replicating cells, that SUZ12 protein levels were significantly downregulated (Fig. 4E), thereby linking expression of these genes to loss of PRC2 function, as modeled by our 4pX-1-*SUZ12*^{KD} cell line (Fig. 3A).

Since hCSCs are characterized by expression of EpCAM, CD44 and CD133 [7,14], we reason that the Wnt⁺/GFP⁺ cell population isolated from HBV replicating cells must share properties with hCSCs. Key characteristic of cancer stem cells (CSCs) is resistant growth to chemotherapeutic drugs. Therefore, we examined growth of sorted Wnt⁺/GFP⁺ cells in presence of chemotherapeutic drugs cisplatin, and sorafenib used in treatment of advanced stage HCC [6]. Sorted GFP⁺ cells were more resistant to cisplatin and sorafenib (Fig. 4E) in comparison to GFP⁻ HBV replicating HepAD38 cells. Furthermore, treatment with DAPT reduced the survival of HBV replicating cells grown in the presence of sorafenib or cisplatin (Fig. 4F). Taken together these result support that EpCAM RIP contributes to chemoresistance of the GFP⁺/Wnt⁺ HBV replicating cells.

EpCAM RIP in liver tumors of HBV X/c-myc bitransgenic mice

To provide evidence that this mechanism of EpCAM RIP observed *in vitro* also occurs *in vivo*, we employed the HBV X/c-myc mouse model of hepatocarcinogenesis [22]. Liver tumors from X/c-myc animals exhibit increased expression of EpCAM, due to downregulation of SUZ12 [19,24]. Here, by immunoblots we observe processing of EpCAM, including the EpCAM CTF fragment, in tumor (T) but not peritumoral (PT) tissue or normal liver (Fig. 5A). Moreover, by qRT-PCR we quantified in liver tumors increased expression of *EpCAM*, *BAMBI*, *CD133* and pluripotency genes, thereby supporting the *in vitro* data (Fig. 4).

A subgroup of HBV-related HCCs express the hCSC gene signature

To determine the clinical relevance of these observations, we designed custom PCR arrays containing probe sets for hCSC markers (*CD44*, *CD133*, *CACNA2D1*, *AFP*), pluripotency

genes (*NANOG*, *OCT4* and *SOX2*), hepatic progenitor/PRC2 target genes (*EpCAM*, *BAMBI*), and proliferation genes (*TYMS*, *MCM6*, *PLK1* and *IGF2*). Employing these custom PCR arrays, we quantified expression profile of these genes in normal human liver (N) and HCC tumor (T) samples from chronic HBV infected patients, including non-tumoral (NT) tissue (Supplementary Fig. 4; Supplementary Table 3).

The data were normalized relative to *GAPDH* and converted to gene-specific z-scores using normal liver (N) samples as reference. Z-scores were visualized as heatmap with samples grouped by manual classification based on expression of *AFP*, *EpCAM*, *BAMBI*, *CD44*, *CD133*, and pluripotency genes (*OCT4*, *SOX2*, *NANOG*). Four subgroups (group I-IV) of HBV-induced HCCs were identified (Fig. 6A): group I, exhibiting increased expression of *EpCAM* and *AFP*, group II, increased expression of *BAMBI*, but not *AFP* and *EpCAM*; group III, exhibiting increased expression of at least four of the hCSC markers (*EpCAM*, *BAMBI*, *CD44*, *CD133*) and pluripotency genes (*SOX2*, *NANOG*, *OCT4*), thus resembling expression of the hCSC-like gene signature identified in Wnt⁺/GFP⁺ HBV replicating cells (Fig. 4C); and group IV that expresses none or only one of the five hCSC markers (*AFP*, *EpCAM*, *BAMBI*, *CD44*, *CD133*).

Importantly, group III tumors display statistically significant induction of *Axin2*, *EpCAM*, *BAMBI* and *CD44* in comparison to normal liver (Fig. 6B). Interestingly, patients in group III, despite having significantly smaller (less than 50 mm) tumors (Table 1) tended to show poor prognosis with reduced overall survival post-surgical resection (Fig. 7A). Hence, the hCSC-like gene signature is a poor prognosis outcome indicator, independent of large tumor size. All groups had increased expression of proliferation genes (*TYMS*, *PLK1* and *MCM6*), *EZH2*, the methyltransferase subunit of *PRC2*, and DNA methyltransferase *DNMT3A* (Fig. 6A; Supplementary Fig. 5).

To validate our observations, we analyzed expression of the hCSC-like gene signature using the transcriptomic dataset of HCCs reported by Boyault et al. [41]. In those studies, G1 liver tumors derived from HBV infected patients, exhibited overexpression of genes characteristic of fetal liver and controlled by parental imprinting, known to be regulated by the PRC2 complex ([42,43], while G4-G6 tumors were associated with β -catenin (*CTNNB1*) activating mutations. Consistent with our results, *EpCAM*, *BAMBI*, *AFP* and *SOX2* exhibited statistically significant induction in G1 tumors and not G4-G6 tumors (Fig. 7B; Supplementary Table 4). Moreover, group III tumors from our study lacked elevated expression of *GLUL*, a marker of mutated *CTNNB1* tumors [44], relative to normal liver. Indeed, group III tumors were devoid of β -catenin gene activating mutations confirmed by DNA sequencing, further confirming our conclusions (Fig. 7C). Thus, activation of Wnt signaling by *EpCAM* RIP as shown in this study, is a poor outcome factor as described previously [44], and contrasts Wnt activation by *CTNNB1* mutations.

Discussion

In this study, we identified a hCSC-like gene signature comprised of genes *EpCAM*, *BAMBI*, *CD44*, *CD133* (known markers of hCSCs) and *NANOG*, *OCT4*, *SOX2* (encoding pluripotency transcription factors). This hCSC-like gene signature is induced by activation

of canonical Wnt signaling following EpCAM RIP in HBV replicating cells. Significantly, in HCCs of chronically HBV infected patients, expression of the hCSC-like gene signature is associated with poor prognosis and reduced overall survival post-surgical resection. We identified this gene signature by mechanistic studies aiming to understand the significance of EpCAM upregulation upon loss of PRC2 function, observed in HBV replicating cells and liver tumors of animals modeling HBx- and HBV-mediated hepatocarcinogenesis [19].

Here we provided evidence that EpCAM, in presence of HBV replication, undergoes RIP, and is inhibited by γ -secretase inhibitor DAPT (Fig. 1). The mechanism of EpCAM RIP in HBV replicating cells as well as in *SUZ12*-knockdown cells (Figs. 2 and 3) is likely linked to loss of PRC2 function, allowing enhanced expression of components regulating γ -secretase activity. Although we have not studied this mechanism, EpCAM RIP generates intracellular EpICD serving as co-activator of β -catenin regulated genes *c-Myc*, *Cyclin D1* [27] and *OCT4* [45,46]. Importantly, EpCAM RIP and Wnt signaling activation occurred both in HepAD38 HBV replication model and HepG2-NTCP HBV infection model (Figs. 1 and 2). Transfection of *EpCAM* siRNA suppressed Wnt signaling (Figs. 2 and 3), thereby demonstrating EpCAM RIP was instrumental in activation of Wnt signaling in HBV replicating cells *in vitro*.

Wnt signaling is essential in liver development and regeneration [7,15] and has a role in HCC pathogenesis [41]. Transcriptomic classification of HCCs [41,44] have identified liver tumors with *CTNNB1* mutations activating Wnt signaling and characterized by good prognosis [41,44], and those of the Wnt-TGF β subclass linked to an aggressive phenotype [44]. Our analyses of liver tumors from X/c-myc mice and clinical HBV-related HCCs identified expression of the hCSC-like gene signature (Figs. 5 and 6 and Supplementary Fig. 5), similar to Wnt⁺ HBV replicating cells, thereby validating our *in vitro* mechanistic results (Figs. 1–4). In our small cohort of HBV-related HCCs (Fig. 6), tumors exhibiting expression of the hCSC-like gene signature strikingly tended to associate with poor prognosis (Fig. 7 and Table 1). We further validated our conclusions by analyzing available transcriptomic HCC datasets [41]. Indeed, select EpCAM RIP signature genes (*EpCAM*, *BAMBI*, *SOX2*) exhibited statistically significant induction in the G1 group of HCCs associated with poor prognosis, and not with the G4-G6 group of tumors associated with *CTNNB1*/ β -catenin activating mutations [41]. DNA sequencing confirmed absence of *CTNNB1* mutations in group III tumors (Fig. 7C). Thus, our results are in accordance with Lachenmayer's data showing Wnt-activated HCCs devoid of *CTNNB1* mutation have aggressive phenotype with resistance to sorafenib [44].

Why the *CTNNB1* subclass has less aggressive phenotype is not understood. Likewise, how various mechanisms of Wnt pathway activation influence expression of Wnt target genes is unknown. For example, in hCSCs, Wnt signaling was associated with elevated expression of *EpCAM*, *BAMBI* and *DKK1* [47,48]. However, these genes were not found to be upregulated in the Wnt-TGF β subclass of HCCs [44]. The important observation of our study is that the subpopulation of HBV replicating cells that undergo EpCAM RIP, Wnt signaling activation and induction of Wnt-responsive genes, also exhibited significantly reduced levels of SUZ12 protein, i.e., loss of PRC2 function (Figs. 3 and 4). In this cellular environment of reduced PRC2 function, we observed increased expression of pluripotency

genes and hCSC marker genes. Thus, our results suggest that reduction of SUZ12 beyond a threshold (Fig. 4D), may be another mechanism that leads to Wnt activation, allowing HBV replicating hepatocytes to reprogram to a hCSC-like gene expression pattern (Fig. 7D).

The significance of the finding that EpCAM RIP induces expression of the Wnt-regulated hCSC-like gene signature in HBV replicating cells and in a subgroup of HBV-related HCCs, is the potential use of γ -secretase inhibitors clinically to suppress EpCAM RIP and the ensuing activation of Wnt signaling. Indeed, γ -secretase inhibitors are in clinical trials for other human cancers [49,50]. Likewise, Wnt signaling inhibitors may also be considered for treatment of HBV-associated HCCs [51]. Furthermore, identification of the hCSC-like gene signature, once validated in a larger cohort of HBV-related HCCs, has the potential to classify patients who will benefit from such intervention. Lastly, the molecular mechanism we identified herein (Fig. 7D) suggests that maintenance of PRC2 function can be explored as a therapeutic strategy to suppress re-expression of EpCAM and the hCSC-like signature, and the cellular reprogramming of chronically infected hepatocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank the French National Biological Resources Centre for frozen human liver tissues, obtained following approved consent from the French Liver Tumor Network *Scientific Committee*. The French Liver Tumor Network is funded by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Agence Nationale de la Recherche (ANR). The authors also thank the Biological Resources Center of Centre Léon Bérard for normal liver tissues obtained following approved consent and ministerial agreement, Drs. Chun-Ju Chang and R.L. Hullinger for critical reading of the manuscript, and Drs. D. Durantel and J. Irudhayaraj for purified HBV virus and mcherry expression vector, respectively. The author(s) acknowledge the use of the Flow Cytometry and Cell Separation Facility of the Bindley Bioscience Center.

Financial support

This work was supported by NIH grant DK044533 to OA, and French grants PAIR-CHC 2009 (contract #2009-143, project ENE-LIVI) from Institute National du Cancer (INCa) to PM. Shared Resources (flow cytometry and DNA sequencing) are supported by NIH grant P30CA023168 to Purdue Center for Cancer Research and NIH/NCRR RR025761

Abbreviations

cccDNA	circular covalently closed DNA
EpCAM	epithelial cell adhesion molecule
EpICD	EpCAM intracellular domain
hCSCs	hepatic cancer stem cells
HBV	hepatitis B virus
HBc	hepatitis B virus core antigen
HCC	hepatocellular carcinoma

PRC2	polycomb repressor complex 2
RIP	regulated intramembrane proteolysis

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Fig. 1. HBV replication induces regulated intramembrane proteolysis of EpCAM

(A) PCR quantification of *EpCAM* mRNA isolated from HepAD38 cells at 0–20 days after onset of HBV replication. (B) Immunoblots of EpCAM, HBV core antigen (HBV replication control) and actin isolated at 0, 5, 10 and 20 days after onset of HBV replication without (–) or with (+) 10 μ M DAPT (γ -secretase inhibitor) treatment for 24 h. (C) ImageJ quantification of EpCAM protein levels from Fig. 1B normalized to actin. (n = 3) (*indicates $p < 0.05$). (D) PCR quantification of *EpCAM* mRNA and immunoblots of EpCAM from uninfected (Day 0) and HBV infected (Day 4) HepG2-NTCP cells without (–) and with (+) 10 μ M DAPT treatment for 24 h.

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Fig. 3. EpCAM RIP in *SUZ12* knockdown cells activates Wnt signaling

(A) Immunoblots of EpCAM and PCR quantification of *EpCAM* mRNA employing 4pX-1^{GIPZ} and 4pX-1-*SUZ12*^{KD} cells without (-) or with (+) HBx, expressed for 18 h by tetracycline removal. (B) Luciferase activity in indicated cell lines following transient co-transfections of TOPflash or FOPflash and Renilla luciferase reporters with *siEpCAM#1* (+) or scrambled siRNA (-) (n = 3). Immunoblot of EpCAM in indicated cell lines following transfection of *siEpCAM#1* (+) or scrambled siRNA (-). (C) Immunoblots of NANOG, OCT4, SOX2 and actin with lysates from indicated cell lines. (D) PCR quantification of pluripotency gene mRNAs in indicated cell lines (n = 3). (E) 4pX-1-*p53*^{KD} cells, transiently transfected (48 h) with TOPGFP plasmid expressing mCherry constitutively, were sorted by FACS. Total RNA isolated from sorted cells was used to quantify mRNA levels of indicated genes. (n = 3, *indicates *p* < 0.05).

Fig. 4. Enhanced expression of hCSC markers and pluripotency genes in GFP⁺/Wnt⁺ HBV replicating cells

(A) FACS of GFP⁺ and GFP⁻ HepAD38 cells, transfected with TOPGFP on Day 0 and Day 10 of HBV replication, with (+) or without (-) *EpCAM* knockdown. (B) Percent GFP⁺ cells quantified from (A), including treatment with 2.5 mM LiCl for 24 h (n = 2) (C) PCR quantification of mRNA expression of indicated genes using total RNA isolated from HBV replicating mCherry⁺/GFP⁺ and mCherry⁺/GFP⁻ HepAD38 cells (n = 3). (D) SUZ12 immunoblots from uninfected HepG2-NTCP cells (Day 0), HBV infected HepG2-NTCP cells (Day 4), HepAD38 cells without HBV replication (Day 0) and FACS sorted HBV replicating HepAD38 cells (Day 20 of HBV replication). (E) Cell viability of sorted GFP⁺ and GFP⁻ HepAD38 HBV replicating cells (5,000 cells from each sorting experiment) plated in triplicates, and treated with indicated concentration of cisplatin or sorafenib. MTS assays were performed 18 h after treatment, according to manufacturer's instructions (n = 3, *indicates $p < 0.05$). (F) Cell viability of HBV replicating HepAD38 cells, on day 10 of HBV replication; 20,000 cells plated in triplicates, and treated with indicated concentration of cisplatin or sorafenib. MTS assays performed 18 h after treatment, according to manufacturer's instructions (n = 3, *indicates $p < 0.05$).

Fig. 5. EpCAM RIP and expression of hCSC-like gene signature in liver tumors from X/c-myc mice

(A) EpCAM immunoblots using lysates from normal mouse liver (2 weeks and 4 months), peritumoral (PT) and tumor (T) liver tissues obtained from 12-month X/c-myc mice. *indicates EpCAM processing fragments. EpCAM CTF detected by 15% SDS PAGE, transferred to polyvinylidene difluoride (PVDF) membrane for immunoblotting with EpCAM antibody in 5% milk. Relative intensity (rel. int.) vs. actin quantified by ImageJ software. A representative assay is shown (n = 3). (B) Boxplot representation of expression of indicated genes in X/c-myc liver tumors. Ct represents $C_{t_{normal}} - C_{t_{tumor}}$. Ct for all samples was calculated by subtracting Ct value of reference gene *GAPDH* from Ct values of gene of interest (*indicates $p < 0.05$).

Fig. 6. Expression of hCSC markers and pluripotency genes in HBV-related HCCs
 (A) Heat map of gene-specific z-scores of HBV-related HCCs (T) relative to normal liver (N) revealed four distinct groups (I-IV). Samples 04T and 03T are outliers and were not assigned to a group. (B) Boxplot representation of expression of indicated genes in HBV-induced liver tumors of Groups I to IV. ΔC_t represents $C_{t_{\text{normal}}} - C_{t_{\text{tumor}}}$. ΔC_t for all samples was calculated by subtracting C_t value of reference gene *GAPDH* from C_t values of gene of interest (*indicates $p < 0.05$).

Fig. 7. Kaplan-Meier curves for overall survival analysis of patients with HBV-related HCCs (A) Survival of patients from Group III vs. Rest, after surgical tumor resection (based on heatmap of Fig. 6A). Log-rank test assessed survivals ($p = 0.07$). (B) Expression of indicated genes in HCC subgroups (G1, G4-G6 and NT, non-tumor) described by Boyault et al. [41]. * $p < 0.06$, ** $p < 0.01$. (C) Mutation status of *CTNNB1* gene and expression level of GLUL, a marker of *CTNNB1* tumors [44], in Group III tumors (n.a., not available). (D) Diagram of working model: in absence of HBV infection, PRC2 represses expression of its target genes. In HBV replicating cells, HBx activates PLK1 which phosphorylates SUZ12 leading to SUZ12 degradation [24]. However, marked downregulation of SUZ12 as shown in Fig. 4D, is associated with generation of a subpopulation of Wnt⁺ HBV replicating cells via EpCAM RIP, and re-expression of the hCSC-like gene signature. (This figure appears in colour on the web.)

Table 1

Clinico-pathological features of 32 chronically HBV infected patients with HCC.

Correlation	Differentiation status (poor)	Differentiation status (well)	AFP >200 ng/ml	Multi-nodularity	Size >50 mm	Satellite nodules	Microvascular invasion
GROUP I (n = 5) vs. Rest	0.07 ($p = 0.69$)	0.04 ($p = 0.82$)	0.13 ($p = 0.48$)	-0.23 ($p = 0.21$)	0.43 ($p = 0.01$)	0.01 ($p = 0.95$)	0.16 ($p = 0.38$)
GROUP II (n = 10) vs. Rest	-0.03 ($p = 0.85$)	-0.22 ($p = 0.22$)	-0.21 ($p = 0.25$)	-0.03 ($p = 0.86$)	0.001 ($p = 0.99$)	0.16 ($p = 0.39$)	-0.17 ($p = 0.36$)
GROUP III (n = 9) vs. Rest	0.06 ($p = 0.72$)	-0.13 ($p = 0.48$)	-0.09 ($p = 0.62$)	0.005 ($p = 0.97$)	-0.35 ($p = 0.05$)	0.13 ($p = 0.46$)	-0.20 ($p = 0.27$)
GROUP IV (n = 8) vs. Rest	-0.09 ($p = 0.62$)	0.33 ($p = 0.06$)	0.20 ($p = 0.26$)	0.21 ($p = 0.23$)	0.001 ($p = 0.99$)	-0.32 ($p = 0.08$)	0.24 ($p = 0.18$)

Correlation was assessed between clinico-bio-pathological parameters and by following the clustering approach. Spearman coefficient of correlation (p value).