 doi:10.20944/preprints201609.0074.v1

Article

Regulation of ERK and AKT Pathways by Hepatitis B Virus X Protein via Notch1 Pathway in Hepatocellular Carcinoma

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Abstract: Hepatitis B virus (HBV) is the dominant risk factor for hepatocellular carcinoma (HCC). HBV X protein (HBx) plays crucial roles in HCC carcinogenesis. HBx interferes with several signaling pathways including Notch1 pathway in HCC. In our study, we found that Notch1 was highly expressed in HCC especially in large HCC. Notch1 and HBx co-localized in HCC and their levels were positively correlated with each other. Notch1 expression was more elevated in HepG2.2.15 than that in HepG2. HBx activated Notch1 pathway in HepG2.2.15. Repression of HBx and Notch1 pathway attenuated the growth of HepG2.2.15. Notch1, ERK and AKT pathways were inhibited after a γ-secretase inhibitor treatment. Dual-specificity phosphatase 1 (DUSP1) and phosphatase and tensin homolog (PTEN) were up-regulated after the γ-secretase inhibitor treatment and Hes1 inhibition. Luciferase reporter assays showed that Hes1 repressed the promoters of DUSP1 and PTEN and this was reverted by γ-secretase inhibitor treatment. Western blotting demonstrated that DUSP1 dephosphorylated pERK and PTEN dephosphorylated pAKT. Collectively, we reported a link among HBx, Notch1 pathway, DUSP1/PTEN, and ERK/AKT pathways, which influenced HCC cell survival and could be a therapeutic target for HCC.

Keywords: hepatocellular carcinoma; hepatitis B virus X protein; Notch1 pathway; ERK; AKT

1. Introduction

Hepatocellular carcinoma (HCC) remains a major health concern worldwide[1]. Hepatitis B virus (HBV) is the dominant risk factor for HCC[2]. It accounts for more than half of all cases[3]. HBV X protein (HBx) is the only expressed HBV protein and plays critical roles in hepatocarcinogenesis[4]. Previous reports have proved that HBx can interfere with many signal-transduction pathways including Hippo, nuclear factor-κB, WNT/β-catenin, and p53 pathways[2,4-7]. However, the molecular mechanisms underlying the hepatocellular carcinogenesis induced by HBx remain ambiguous.

Notch pathway plays crucial roles in organogenesis and morphogenesis and influences various biological processes in apoptosis, proliferation, and differentiation[8]. Emerging evidences demonstrate that dysregulation of Notch pathway is associated with various types of malignancies[9,10]. Persistent activation of Notch pathway leads to liver malignancies[11]. The link between HBx and Notch pathway has been reported[12,13]. Previous researches have reported that HBx activates Notch1 pathway which further up-regulates ERK and AKT pathways to promote cell proliferation[14,15]. However, the detail molecular mechanisms whereby Notch1 activates ERK and AKT pathways in HCC remain unresolved.

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In this study, we further explored the link between HBx and Notch1 in HCC and elucidated the molecular mechanisms underlying Notch1/ERK and Notch1/AKT activations by HBx.

2. Results

2.1. Expression of Notch1 in human liver and HCC tissues

Immunohistochemistry analysis showed that Notch1 was highly expressed in HCC tissues. Notch 1 also exhibited expression in adjacent nontumor and cirrhosis tissues, however, its expression was significantly lower than that in HCC tissues (P<0.01). Moreover, the expression of Notch1 in normal liver tissues was significantly lower than that in the above-mentioned tissues (P<0.001) (Figure 1A,B). Western blotting analysis showed the similar results (Figure 1C). In most of HCC tissues, the expression of Notch1 was much elevated than that in the other tissues. Notch1 was all lowly expressed in normal liver tissues.

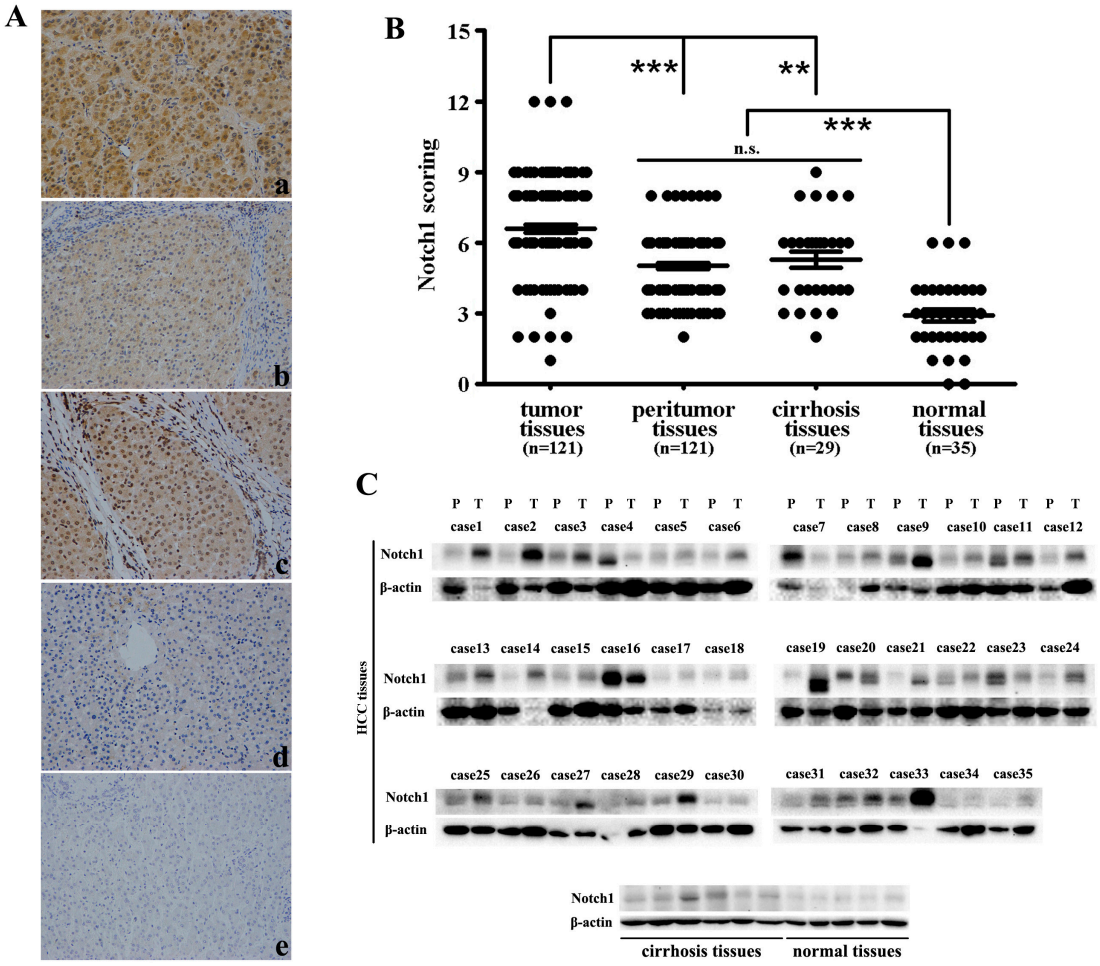

Figure 1. Notch1 was highly expressed in HCC tissues. (**A**) Immunohistochemical staining of Notch1 in HCC (a), adjacent nontumor (b), cirrhosis (c) and normal liver tissues (d). (e) Negative control. (**B**) The immunohistochemistry score of Notch1 in each immunostained liver section. (**C**) Western blotting of Notch1 in HCC, adjacent nontumor, cirrhosis and normal liver tissues. (** $p < 0.01$, *** $p < 0.001$; n.s. means no significance)

Specifically speaking, in 121 HCC tissues, 19 of them were lowly expressed and 102 of them were highly expressed. In 121 corresponding peritumor tissues, 59 of them were lowly expressed and 62 of them were highly expressed (P <0.001) (Table 1).

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Table 1. Expression of Notch1 in HCC and peritumor liver tissues

	Cases tested	Relative Notch1 expression		*P* value
		Low	High	
Tumor tissues	121	19	102	<0.001
Peritumor tissues	121	59	62	

Table 2 showed the correlation between the factors and clinicopathologic parameters in 121 HCC patients. As shown in the table, none of gender, age, and AFP level was related to Notch1 expression. The level of Notch1 was significantly more elevated in HBV positive patients (P<0.001), cirrhosis patients (P<0.05), and large HCC patients (P<0.05).

Table 2. Correlation between the factors and clinicopathologic parameters in HCC patients (n=121)

Clinicopathological variables	Relative Notch1 expression		*P* value
	Low	High	
Gender			
Male (n=91)	16	75	0.322
Female (n=30)	3	27	
Age (years)			
≤50 (n=49)	9	40	0.506
>50 (n=72)	10	62	
HBV			
Negative (n=13)	7	6	<0.001
Positive (n=108)	12	96	
AFP (ug/L)			
≤20 (n=41)	8	33	0.410
>20 (n=80)	11	69	
Cirrhosis			
No (n=29)	8	21	0.044
Yes (n=92)	11	81	
Tumor size (cm)			
≤5 (n=54)	13	41	0.023
>5 (n=67)	6	61	

2.2. Co-localization and relation of Notch1 with HBx in HCC tissues and HepG2.2.15 cell line

Previous reports showed the relationship of Notch1 with HBx. Here, we found the co-localization of the two proteins. Immunohistochemistry results showed that Notch1 and HBx co-expressed in HCC tissues (Figure 2A). Figure 2B showed the correlation of expression levels of Notch1 and HBx. The Spearman's rho was 0.584, which meant that Notch1 levels were positively correlated with HBx expression in the 108 HBV-positive HCC tissues. Figure 2C showed that HBx was stained green whereas Notch1 was stained red in HepG2.2.15 cells. The yellow staining in dual-labeling experiments indicated overlapping areas of red and green fluorescent labels, suggesting co-expression of Notch1 with HBx in HepG2.2.15 cells.

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2.3. Regulation of Notch1 by HBx in HepG2.2.15 cells

Since the relation of HBx with Notch1 was found in HCC tissues and cells, we wonder whether HBx could regulate regulate the expression of Notch1 in HepG2.2.15 cells in vitro. To verify this hypothesis, we compared the expression of HBx as well as some members of Notch1 pathway between HepG2 and HepG2.2.15, and between HepG2.2.15- SiCtrl and HepG2.2.15-SiHBx cells. Western blotting and PCR confirmed that HepG2 didn't express HBx whereas HBx gene and protein were expressed in HepG2.2.15 (Figure 3A,B). Then we tested the expression of some members of Notch1 pathway such as Jagged1, Notch1 and Hes1. Western blotting showed that the protein level of the three proteins was much more elevated in HepG2.2.15 than in HepG2 cells (Figure 3B). Then we knocked down HBx expression of in HepG2.2.15 by HBx specific siRNA. Western blotting and PCR confirmed that HBx expression was significantly decreased by siRNA (Figure 3C,D).

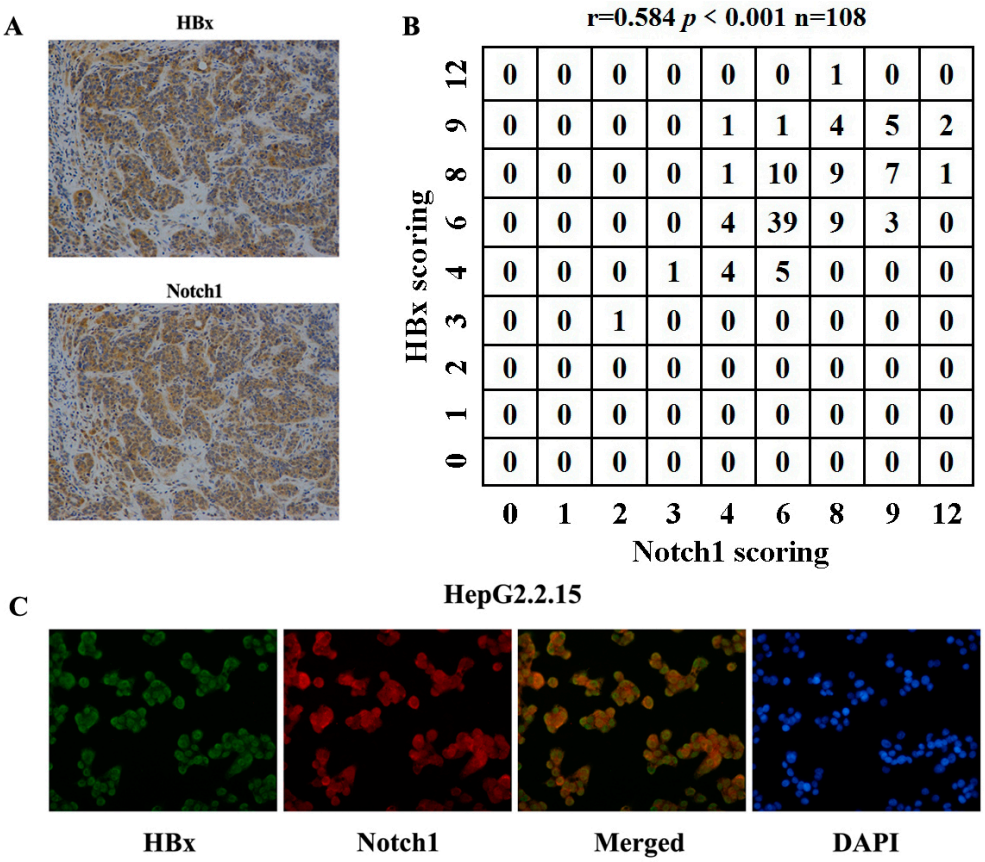

Figure 2. Notch1 and HBx co-expressed in HCC tissues and HepG2.2.15 cells. (**A**) Co-localization of Notch1 and HBx detected by immunohistochemical staining. (**B**) Correlation of expression levels of Notch1 and HBx in 108 HBV-positive HCC tissues. The Spearman's rho =0.584, P<0.0001. (**C**) Co-expression of Notch1 with HBx in HepG2.2.15 detected by double-fluorescence immunostaining.

Western blotting showed that the decrease of HBx expression led to an accordant decrease of Notch1 and Hes1 in HepG2.2.15 (Figure 3D), verifying that HBx could regulate the expression of Notch1 in vitro.

2.4. Suppression of HBx and Notch1 pathway inhibited the growth of HepG2.2.15

Since our results indicated that Notch1 was highly expressed in HCC tissues especially in large HCC tissues and Notch1 pathway was regulated by HBx, we therefore determined whether inhibition of Notch1 pathway would attenuated cell growth. To inhibit the Notch1 pathway, we used the γ-secretase inhibitor DAPT. HepG2.2.15 cells were treated with increasing concentrations of DAPT for 1, 2, 3, 4d and the cell viability was evaluated by CCK-8 assay. As shown in Figure 3E,

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inhibition of HBx attenuated cell growth of HepG2.2.15. Increasing concentration and treatment time of DAPT resulted in a progressive inhibition of HepG2.2.15 cell viability (Figure 3F). At 1d, DAPT treatment with various concentrations did not produce any reduction in cell viability. Nevertheless, DAPT treatment for more than 2d triggered a significant time- and dose-dependent decrease in cell viability of HepG2.2.15 cells. We ultimately selected DAPT treatment concentration of 20μM for further studies.

2.5. Inhibition of Notch1, ERK and AKT pathways after DAPT treatment in HepG2.2.15

HepG2.2.15 cells treated with 20μM DAPT for 1 and 6h were assessed for levels of inhibition of Notch1 pathway by Western blotting analysis of Jagged1, Notch1, NICD, and Hes1 expression. DAPT treatment

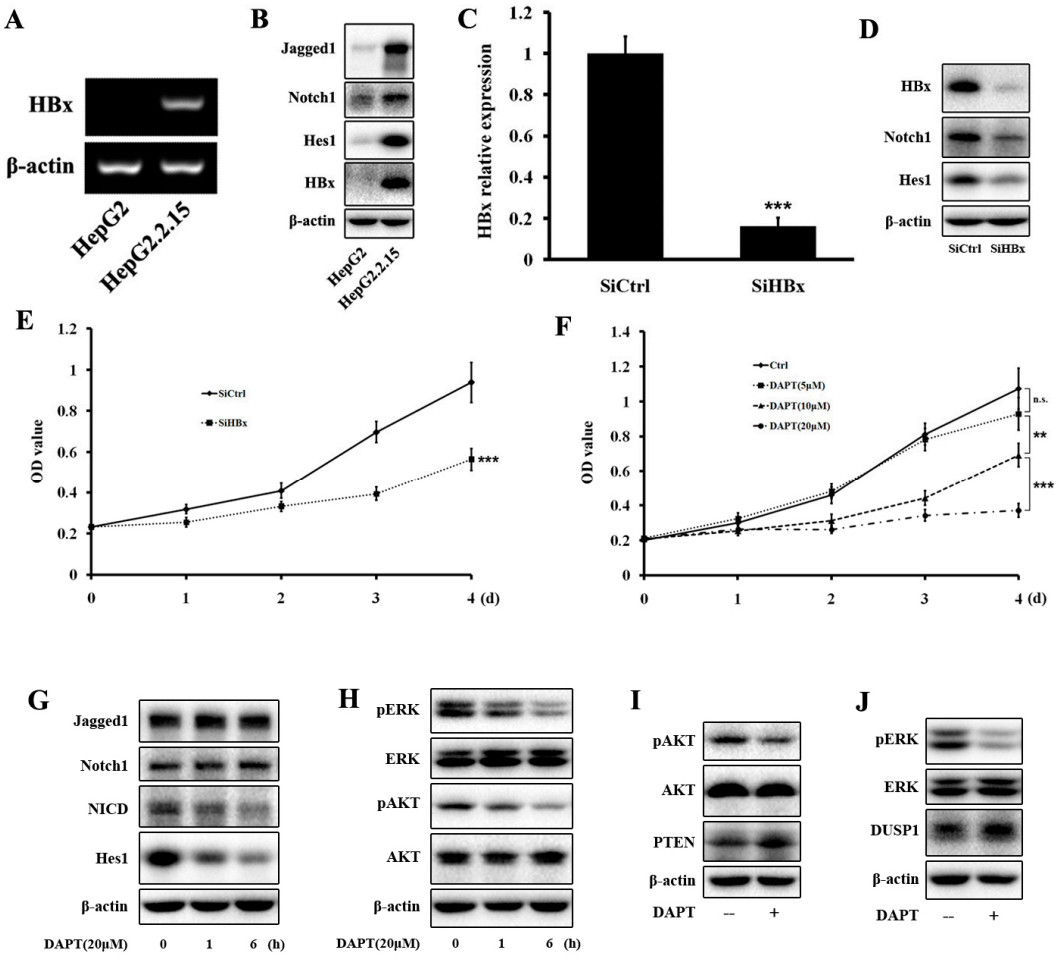

Figure 3. HBx activated Notch1 pathway and DAPT attenuated cell growth via inhibition of ERK and AKT pathways. HBx gene (**A**) and protein (**B**) were expressed in HepG2.2.15. (B) Notch1 pathway was activated in HepG2.2.15. (**C**) HBx was knocked down by SiRNA and its mRNA level was tested by qRT-PCR. (**D**) Notch1 pathway was inhibited after SiHBx. (**E**) Cell proliferation of HepG2.2.15 was inhibited after SiHBx. (**F**) Cell proliferation of HepG2.2.15 was inhibited after DAPT treatment. (**G**) Alteration of expression of Notch1 pathway after DAPT treatment. (**H**) Expressions of pERK and pAKT were decreased after DAPT treatment. (**I**) and (**J**) Expressions of DUSP1 and PTEN were increased after DAPT treatment. Data were collected in at least three independent experiments. (** $p < 0.01$, *** $p < 0.001$; n.s. means no significance)

significantly decreased the amount of NICD and Hes1 in a time-dependent manner, while did not have any effect on Jagged1 and Notch1 (Figure 3G). Our data showed that DAPT treatment greatly inhibited cell growth of HepG2.2.15, so we tested expression of ERK and AKT pathways which are

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closely related to cell proliferation. Western blotting analysis indicated that greatly reduced the amount of pERK and pAKT in a time-dependent manner, while did not have any effect on total ERK and AKT (Figure 3H).

2.6. Up-regulation of DUSP1 and PTEN after DAPT treatment in HepG2.2.15

Previous reports have verified the regulatory circuit linking Notch1 pathway with DUSP1 expression and ERK activity[16]. The linking Notch1 pathway with PTEN and AKT activity has also been found[17]. Our data showed that DUSP1 and PTEN were up-regulated after DAPT treatment in HepG2.2.15 by western blotting (Figure 3I,J).

2.7. Up-regulation of DUSP1 and PTEN after SiHes1 in HepG2.2.15

We wanted to test the effect of Hes1 inhibition on DUSP1 and PTEN levels. Treatment of HepG2.2.15 cells with siRNA targeting Hes1 mRNA (SiHes1) effectively reduced Hes1 mRNA level (Figure 4A). Cell growth of HepG2.2.15 was inhibited after SiHes1 (Figure 4B). Then we found that mRNA levels of DUSP1 and PTEN were greatly increased after SiHes1 (Figure 4C,D). Western blotting analysis showed the same results. Hes1 protein level was significantly reduced and the protein levels of DUSP1 and PTEN were significantly elevated (Figure 4E).

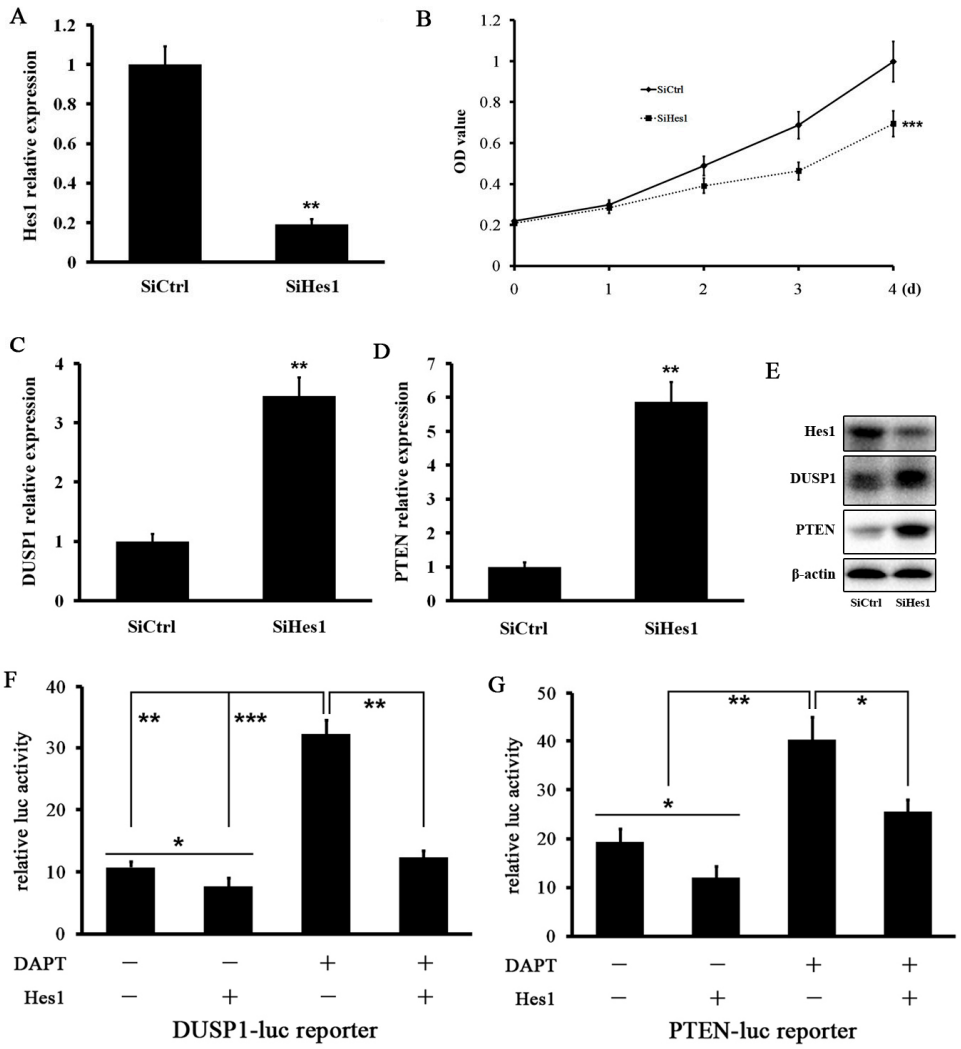

Figure 4. Hes1 repressed the expressions of DUSP1 and PTEN. (**A**) Hes1 mRNA level was effectively reduced after SiHes1. (**B**) Cell growth of HepG2.2.15 was inhibited after SiHes1. (**C**) DUSP1 mRNA level was increased after SiHes1. (**D**) PTEN mRNA level was increased after SiHes1. (**E**) Protein levels of DUSP1

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and PTEN were increased after SiHes1. (**F**) and (**G**) Luciferase activity of HepG2.2.15 transfected with a DUSP1-luc reporter or PTEN-luc reporter together with a plasmid expressing HES1 or its corresponding empty control. Cells were treated with DAPT or its vehicle. Data were collected in at least three independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

2.8. Hes1 directly bound and repressed the promoters of DUSP1 and PTEN in HepG2.2.15

On the basis of our above data, we wanted to test if Hes1 could repress DUSP1 and PTEN. We performed

DUSP1 and PTEN promoter assays using a luciferase reporter. The basal activity of the DUSP1 and PTEN promoters was reduced by Hes1. Treatment of HepG2.2.15 cells with DAPT induced the DUSP1 and PTEN promoters, and this was reversed by cotransfection of Hes1 (Figure 4F,G). These results further reinforce our previous findings that DAPT up-regulated DUSP1 and PTEN expressions by down-regulating HES1.

2.9. Elevated pERK/pAKT after SiDUSP1/SiPTEN in HepG2.2.15

We wanted to test the effect of DUSP1 inhibition on ERK phosphorylation. Treatment of HepG2.2.15 cells with siRNA targeting DUSP1 mRNA (SiDUSP1) effectively reduced DUSP1 mRNA level and protein levels, and this resulted in elevated pERK level (Figure 5A,B). Then we evaluated the effect of PTEN inhibition on AKT phosphorylation. Treatment with SiPTEN effectively decreased PTEN mRNA level and protein levels, and this induced an increase of pAKT level (Figure 5C,D). The above findings demonstrated that DUSP1 dephosphorylated pERK and PTEN dephosphorylated pAKT.

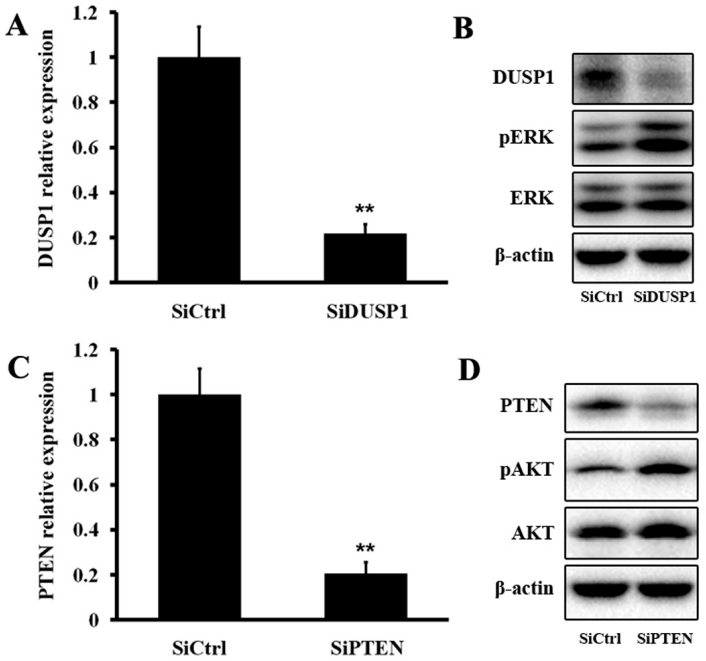

Figure 5. DUSP1 regulated ERK pathway and PTEN regulated AKT pathway. (**A**) and (B) DUSP1 mRNA and protein levels were significantly reduced after SiDUSP1. (**B**) Expression of pERK was increased after SiDUSP1. (**C**) and (**D**) PTEN mRNA and protein levels were significantly reduced after SiPTEN. (**D**) Expression of pAKT was increased after SiPTEN. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3. Discussion

Notch1 is overexpressed in HCC[14]. Consistent with the results of previous studies, we found that Notch1 expression in tumor tissues was much more elevated than that in peritumor, cirrhosis,

and normal tissues by IHC and western blotting analyses. Previous reports have showed the co-localization of Notch1 with HBx[14]. In accordance with these findings, IHC and confocal analyses demonstrated that Notch1 morphologically co-localized with HBx in HCC tissues and HepG2.2.15 cells. What's more, there existed a positive correlation between Notch1 and HBx expression. It has been proved that HBx activates Notch1 pathway. We also found that Notch1 pathway was up-regulated by HBx. Cell growth of HepG2.2.15 was inhibited by treatment of SiHBx or DAPT, which meant that HBx stimulated cell proliferation via Notch1 pathway. Previous report has shown that HBx inhibition attenuates the phosphorylations of ERK and AKT in HepG2.2.15[15]. In our study, we found that inhibition of Notch1 pathway down-regulated the phosphorylated levels of ERK and AKT.

Previous studies have proved that Hes1, the key member of Notch1 pathway, represses expression of DUSP1 which is active against pERK in non-small cell lung carcinoma[16]. Another paper has shown that Hes1 decreases expression of PTEN which is the negative regulator of AKT pathway in T-cell leukemia[17]. Thus, we hypothesized that Notch1 pathway could influence expressions of DUSP1 and PTEN and further exert impact on ERK and AKT pathways in HepG2.2.15. To verify the hypothesis, we performed PCR, western blotting and luciferase assays. We found that mRNA and protein levels of DUSP1 and PTEN were increased after Hes1 inhibition. To investigate the mechanisms that link inhibition of Notch1 pathway with dephosphorylations of ERK and AKT, we analyzed the transcriptional changes induced by DAPT. The promoter inductions of DUSP1 and PTEN after DAPT treatment were confirmed in HepG2.2.15. Previous results have shown that ERK and AKT pathways were the important pathways to regulate Notch1 pathway downstream of HBx. However, the direct link between Notch1 and ERK /AKT pathways has been unrevealed[15].

Hes1 is a famous transcriptional regulator of multiple genes[18,19]. Based on our finding that Hes1 level was reduced upon DAPT treatment of HepG2.2.15, we hypothesized that dephosphorylation of ERK or AKT induced by DAPT could be mediated by Hes1-mediated decrease of DUSP1 or PTEN. Our luciferase reporter assays showed that Hes1 was a negative controller of DUSP1 and PTEN. It directly repressed DUSP1 and PTEN promoters and this was reverted by DAPT treatment. Then we found that increased levels of pERK and pAKT were induced by inhibitions of DUSP1 and PTEN, respectively. This could explain the decrease in ERK and AKT phosphorylations upon DAPT treatment.

Taken together, our studies explored a direct link among HBx, Notch1 pathway, DUSP1/PTEN, and ERK/AKT pathways (Figure 6). We found that HBx activated Notch1 pathway to promote cell growth and this was correlated with the capacity of Hes1 to increase ERK/AKT activity through decrease of DUSP1/PTEN. Nevertheless, we could not exclude that other molecular mechanisms might take part in causing the effects of DAPT. The underlying mechanisms need to be further elucidated.

4. Materials and Methods

4.1. Patients and clinical specimens

A total number of 121 human liver tissue samples were collected from patients who underwent surgical resections at Hepatic Surgery Centre, Tongji Hospital of Huazhong University of Science and Technology (Wuhan, China). Detailed clinicopathologic parameters were listed in Table 2. The procedure of human specimen collection was approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology (HUST), and the study was conducted according to the Declaration of Helsinki Principles.

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4.2. Cell lines, cell culture, and reagents

The human hepatoma cell line HepG2 and and HBV genome-transfected HepG2 (HepG2.2.15) were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). Cells were cultured in high glucose DMEM medium (Gibco, Carlsbad, CA, USA) containing 10 % FBS (Gibco). Dimethyl sulphoxide (DMSO) and the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DAPT was dissolved in DMSO.

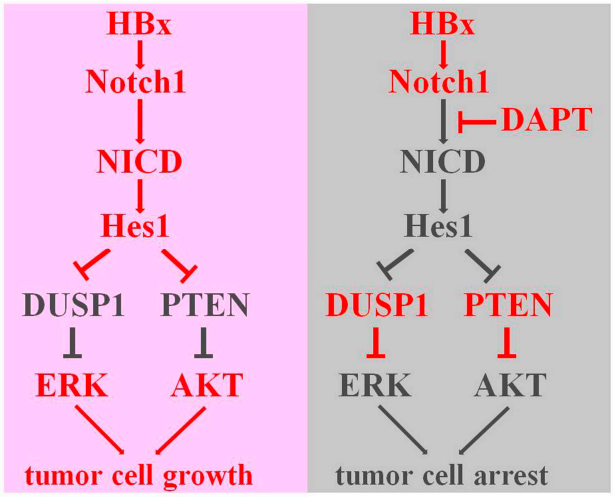

Figure 6. Schematic illustrations of the role of HBx and Notch1pathway in HCC proliferation explored in this study. See the discussion part for the detailed explanation.

4.3. Immunohistochemistry analysis

Immunohistochemistry (IHC) analysis was performed as described previously[20]. The HBx and Notch1 primary antibodies were purchased from Merck-Millipore (Billerica, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively.

4.4. Western blotting

Western blotting was performed as described previously[21]. The primary antibodies and their sources were as follows: Notch1, Hes1, NICD, pERK, ERK, and pAKT (Cell Signaling Technology, Beverly, MA, USA); Jagged1, β-actin, AKT, and DUSP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HBx (Merck-Millipore, Billerica, MA, USA); and PTEN (Proteintech Group, Chicago, IL, USA). The horseradish peroxidase conjugated secondary antibodies were all purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA).

4.5. Double immunofluorescent analysis

Double-fluorescence immunostaining was performed as described previously[22,23]. Double-labeled immunofluorescence was used to detect HBx and Notch1 simultaneously. All sections were analyzed using confocal laser-scanning microscopy on a Nikon Digital ECLIPSE C1 system (Nikon Corporation, Japan).

4.6. Reverse transcription and real-time quantitative PCR analysis

PCR was performed as described previously[24]. The primer sequences for HBx were as follows: sense 5′-GGCTGCTAGGCTGTGCTGCC-3′, anti-sense

 doi:10.20944/preprints201609.0074.v1

5′-GTTCCTGTGGGCGTTCACGG-3′. The images of the bands were acquired using the Alpha Innotech Fluorochem Imaging system. The real-time PCR primers used were listed in Table 3.

Table 3. Primer sequences for real-time PCR

Gene	Primer sequences
HBx	F:5'-CAC CTC TCT TTA CGC GGA CT-3'
	R: 5'-GGT CGT TGA CAT TGC AGA GA-3'
Hes1	F: 5'-AAG AAA GAT AGC TCG CGG CAT-3'
	R: 5'-CCA GCA CAC TTG GGT CTG T-3'
DUSP1	F: 5'-CCA GTA CAA GAG CAT CCC TGT-3'
	R: 5'-AGT GGA CAA ACA CCC TTC CTC-3'
PTEN	F:5'-AGC GTG CAG ATA ATG ACA AGG-3'
	R: 5'- TGG ATC AGA GTC AGT GGT GTC-3'
β-actin	F: 5'-CAA GGC CAA CCG CGA GAA GAT-3'
	R: 5'-CCA GAG GCG TAC AGG GAT AGC AC-3'

4.7. Transient RNA interference

Small interfering RNA (siRNA) duplexes targeting human HBx, Hes1, DUSP1, PTEN sequence and scrambled siRNA were designed as described previously[5,16,25,26]. All siRNAs were synthesized by Ribobio (Guangzhou, China). Transfection of the siRNA duplexes was performed by jetPRIME (Polyplus-transfection SA, Illkirch, France) according to the manufacturer's instructions.

4.8. Cell viability assay

Cell viability assay was performed as described previously[27]. HepG2.2.15 (2×103 cells per well) cells were seeded and cultured in 96-well plates for the indicated time periods. Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was added in the plates for 2 h to test the optical density (OD) value at 450 nm.

4.9. Transcriptional response assay

Luciferase assay was done performed as described previously[28]. The cell lysates was subjected to Luciferase assay using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. DUSP1-luc reporter together with a Hes1 expression vector was transfected as reported previously[29,30]. The relative luciferase activities were normalized against Renilla luciferae used as internal control and were determined by a Glomar 20/20 Illuminometer (Promega).

4.10. Statistical analysis

Data were analyzed using GraphPad Prism 5.0 (La Jolla, CA, USA) or SPSS 13.0 (Chicago, IL, USA). All experiments were performed at least three independent times, and the results were presented as the mean ± SEM. Comparisons between the different groups were evaluated using one-way analysis of variance, and $p<0.05$ was considered statistically significant.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 81272421).

Author Contributions: Bo Liao and Hong-Hao Zhou performed experiments. Bo Liao analyzed the data and wrote the manuscript. Hui-Fang Liang provided technical support. Chang-Hai Li obtained funding, designed this experiment and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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