ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2018 September 14; 24(34): 3813-3964

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 34 September 14, 2018

META-ANALYSIS

CASE REPORT

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Mitsushige Sugimoto, MD, PhD, Associate Professor, Division of Digestive Endoscopy, Shiga University of Medical Science Hospital, Otsu 520-2192, Japan

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORIAL OFFICE
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

PUBLICATION DATE
September 14, 2018

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

World J Gastroenterol 2018 September 14; 24(34): 3861-3870

DOI: 10.3748/wjg.v24.i34.3861

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Delta-like ligand 4 in hepatocellular carcinoma intrinsically promotes tumour growth and suppresses hepatitis B virus replication

Areerat Kunanopparat, Jiraphorn Issara-Amphorn, Asada Leelahavanichkul, Anapat Sanpavat, Suthiluk Patumraj, Pisit Tangkijvanich, Tanapat Palaga, Nattiya Hirankarn

Areerat Kunanopparat, Jiraphorn Issara-Amphorn, Asada Leelahavanichkul, Nattiya Hirankarn, Center of Excellence in Immunology and Immune Mediated Diseases, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Anapat Sanpavat, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Suthiluk Patumraj, Center of Excellence for Microcirculation, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Pisit Tangkijvanich, Research Unit of Hepatitis and Liver Cancer, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

ORCID number: Areerat Kunanopparat (0000-0001-6311-7255); Jiraphorn Issara-Amphorn (0000-0002-7421-2749); Asada Leelahavanichkul (0000-0002-5566-6403); Anapat Sanpavat (0000-0002-6425-3379); Suthiluk Patumraj (0000-0002-8346-2205); Pisit Tangkijvanich (0000-0002-2926-8671); Tanapat Palaga (0000-0001-8734-6626); Nattiya Hirankarn (0000-0003-2224-6856).

Author contributions: Kunanopparat A, Palaga T and Hirankarn N conceived and designed experiments; Kunanopparat A, Issara-Amphorn J, Leelahavanichkul A, Patumraj S and Tangkijvanich P conducted the experiments; Kunanopparat A and Sanpavat A analysed the data; Kunanopparat A, Palaga T and Hirankarn N wrote the manuscript.

Supported by National Research Council of Thailand 2013; the Ratchadaphiseksomphot Matching Fund from the Faculty of Medicine, Chulalongkorn University; the International Research Integration, Chula Research Scholar, Ratchadaphisek somphot Endowment Fund, Center of Excellence in Immunology and Immune-mediated Diseases; and the Rachadapisaek Sompote Post-Doctoral Fund, Chulalongkorn University.

Institutional review board statement: The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

Institutional animal care and use committee statement: All protocols were carried out in accordance with relevant guidelines and regulations.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Correspondence to: Nattiya Hirankarn, MD, PhD, Lecturer, Professor, Center of Excellence in Immunology and Immune Mediated Diseases Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. nattiya.H@chula.ac.th
Telephone: +66-2-2564132
Fax: +66-2-2525952

Received: May 25, 2018
Peer-review started: May 25, 2018

First decision: June 21, 2018
Revised: July 5, 2018
Accepted: July 16, 2018
Article in press: July 16, 2018
Published online: September 14, 2018

Abstract

AIM

To investigate the role of Delta-like ligand 4 (DLL4) on tumour growth in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) *in vivo*.

METHODS

We suppressed *DLL4* expression in an HBV expressing HCC cell line, HepG2.2.15 and analysed the growth ability of cells as subcutaneous tumours in nude mice. The expression of tumour angiogenesis regulators, VEGF-A and VEGF-R2 in tumour xenografts were examined by western blotting. The tumour proliferation and neovasculature were examined by immunohistochemistry. The viral replication and viral protein expression were measured by quantitative PCR and western blotting, respectively.

RESULTS

Eighteen days after implantation, tumour volume in mice implanted with shDLL4 HepG2.2.15 was significantly smaller than in mice implanted with control HepG2.2.15 ($P < 0.0001$). The levels of angiogenesis regulators, VEGF-A and VEGF-R2 were significantly decreased in implanted tumours with suppressed *DLL4* compared with the control group ($P < 0.001$ and $P < 0.05$, respectively). Furthermore, the suppression of DLL4 expression in tumour cells reduced cell proliferation and the formation of new blood vessels in tumours. Unexpectedly, increased viral replication was observed after suppression of DLL4 in the tumours.

CONCLUSION

This study demonstrates that DLL4 is important in regulating the tumour growth of HBV-associated HCC as well as the neovascularization and suppression of HBV replication.

Key words: Hepatocellular carcinoma; Notch signalling; Delta-like ligand 4; HepG2.2.15

Core tip: We demonstrated that Delta-like ligand 4 (DLL4) is important for tumour growth of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) in a xenograft model. We found that the level of angiogenesis regulators, VEGF-A and VEGF-R2 were significantly decreased in HCC xenograft tumours with suppressed DLL4 compared with the control group. Consistent with these findings, the suppression of DLL4 expression in the tumour cells reduced cell proliferation and the formation of new blood vessels in the tumour. Furthermore, this is the first report that DLL4 in an HBV expressing HCC cell line plays a key role in regulating tumour growth, angiogenesis, and viral replication in a mouse model of xenograft transplantation.

Kunanopparat A, Issara-Amphorn J, Leelahavanichkul A, Sanpavat A, Patumraj S, Tangkijvanich P, Palaga T, Hirankarn N. Delta-like ligand 4 in hepatocellular carcinoma intrinsically promotes tumour growth and suppresses hepatitis B virus replication. *World J Gastroenterol* 2018; 24(34): 3861-3870 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i34/3861.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i34.3861

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-associated mortality. Approximately 80% of HCC is associated with chronic hepatitis viral infections[1]. Hepatitis B virus (HBV) infection is the most prevalent cause of HCC in developing countries. Although an HBV vaccine has successfully prevented HBV infection, there are still a large number of chronic hepatitis B patients who are at a high risk (maximum 100-fold increase over healthy individuals) of developing liver cancer[2,3]. The molecular mechanisms of HBV-associated HCC are poorly understood[4]. To date, sorafenib is the recommended drug for the treatment of HCC patients. However, the therapeutic outcome is still limited because liver cancer is often detected at advanced stages[5]. Therefore, a better understanding of the molecular mechanisms of tumour initiation and progression is needed for the further development of HCC therapy.

Notch signalling is an evolutionarily conserved pathway that regulates cell fate decision, embryonic development, tissue homeostasis, differentiation, proliferation, and apoptosis[6,7]. In mammals, the Notch pathway comprises of four Notch receptors (Notch1, 2, 3, 4) and five Notch ligands (Jagged1, 2, and DLL1, 3, 4). Activation of Notch signalling requires contact between a Notch ligand from the signal sending cells and a receptor on signal receiving cells to activate proteolytic cleavage and the subsequent translocation of the Notch intracellular domain to the nucleus where it translates target genes[8]. Dysregulation of Notch signalling has been reported in many types of cancer as either a tumour suppressor or tumour promoter depending on the type of cancer[9-11]. In HCC, the role of Notch signalling is still controversial. Many studies reported that Notch receptors were highly expressed in HCC compared with the adjacent human tumour tissue and that tumour growth was suppressed after the inhibition of Notch either by a gamma secretase inhibitor or by suppression of Notch target genes[12-16]. Several studies have also suggested that Notch is a tumour suppressor in HCC[15,17-19]. However, more evidence supports the pro-tumourigenic role of Notch in HCC carcinogenesis and progression, especially in HBV-associated HCC[20,21]. We previously reported that HBV regulatory protein HBx promoted HBV-associated HCC

proliferation through Delta-like ligand 4 (DLL4) *via* the NF-κB pathway in HepG2, an HBV expressing HCC cell line[22].

Strong evidence indicates that DLL4 regulates angiogenesis and controls the balance of endothelial tip and stalk cell differentiation induced by VEGF[23]. DLL4 is highly expressed in tumour endothelial cells for tumour angiogenesis, which is the primary signal for tumour progression[24]. The inhibition of DLL4 in tumour endothelial cells suppressed tumour growth by inducing non-productive angiogenesis[25]. Currently, a DLL4 neutralizing antibody has been developed and is being tested in a clinical trial for anticancer therapy in various cancers[26,27]. However, the effect of DLL4 inhibition in HCC has not been explored. In this study, we investigated the role of DLL4 on tumour growth in HCC associated with HBV in a xenograft model and detailed the molecular mechanism of HCC.

MATERIALS AND METHODS

Cell culture

The HBV-expressing HCC cell line (HepG2.2.15) and the HepG2 cell line were obtained from Professor Antonio Bertoletti [Singapore Institute for Clinical Sciences at Agency for Science, Technology and Research (A*Star)]. Cells were cultured in high glucose DMEM medium (Gibco, Carlsbad, CA, United States) supplemented with 10% foetal bovine serum (Gibco), 150 μg/mL of G418 (Gibco), and 1% of penicillin-streptomycin (Invitrogen, Carlsbad, CA, United States). Cultures were maintained at 37 ℃ in a 5% CO_2 humidified incubator

Generation of stable DLL4 knockdown cell lines

HepG2.2.15 cells line was transfected with a set of DLL4 shRNA (Origene Technologies, MD, United States) targeting four shDLL4 cassettes in the pGFP-V-RS Vector (TG304977). The transient transfection used Lipofectamine 2000 (Invitrogen) at 2.5 μL for 1 μg of shRNA vector into 1×10^5 cells per well in a 12-well plate. After 48 h, DLL4 mRNA expression was determined in transfected cells. The highest efficacy of shRNA (5′-ACCAGAAGAAGGAGCTGGAAGTGGACTGT-3′) vector was used to generate stably transfected cells. For stably transfected cell lines, transiently transfected cells were plated into 96-well plates by limiting dilution and selected by the addition of 0.3 μg/mL puromycin to the culture medium for 4-5 wk. Puromycin-resistant clones with suppressed DLL4 were expanded, and DLL4 expression was analysed by western blot analysis and compared with the control. The clones with the highest degree of DLL4 suppression were used for tumour xenografts.

In vivo study and tumour xenograft

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. All protocols were carried out in accordance with relevant guidelines and regulations. Male BALB/cMlac-nu mice aged four weeks were purchased from the National Laboratory Animal Center (Mahidol University, Thailand) and were acclimatized for two weeks before experimentation. Mice were maintained under 12 h light-dark cycle with 50% humidity and with free access to food and water. The shDLL4 HepG2.2.15 and control HepG2.2.15 cells were trypsinized at a concentration of 1×10^7 cells/mL. One millilitre of the cells was centrifuged and resuspended in 100 μL of Matrigel (Corning, NY, United States). The cell suspension was subcutaneously injected into the back left and right flanks of nude mice (n = 4-6). The tumour volume (cm^3) was measured every three days until 18 d and 30 d using Vernier calipers and calculated using the formula: (length × width^2)/6. The mice were weighed every three days and monitored for activity and mortality. All animals were euthanized by barbiturate overdose for tumour collection.

Western blotting analysis

Total cell lysates were prepared in RIPA buffer (Cell Signaling Technology, MA, United States) containing protease inhibitor cocktail (Pierce, Thermo Fisher Scientific, MA, United States). After sonication, 20 μg of cell lysates were blotted and probed with primary antibodies to anti-DLL4, anti-cleaved Notch 1, anti-VEGFR2, anti-β actin (Cell Signaling Technology; 1:1000), anti-VEGF, anti-PreS1 HBV antigen, and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, United States; 1:1000). Peroxidase-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology) and goat anti-mouse immunoglobulin (Cell Signaling Technology) were used as secondary antibodies. Immunoblot detection was performed using Super Signal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Fisher Scientific). The protein intensity was estimated by the densitometry of scanned immunoblot bands using Image Studio Lite version 5.2 software (LI-COR Biosciences).

Immunohistochemistry analysis

After the end of the experiment, the tumours were collected, fixed in 10% formalin solution, and embedded in a paraffin block. The tissue sections were cut with a microtome to obtain 4 μm thick paraffin sections, then deparaffinized and rehydrated in a series of xylenes and alcohols followed by retrieval of the antigenic epitopes. Antigen retrieval was performed in citrate buffer (pH 6, 100 ℃ for 20 min). The tissue sections were treated with 3% H_2O_2 for 15 min and blocked with normal serum for 30 min, then incubated with primary antibody in a humidity chamber at 4 ℃ overnight. The primary antibodies included anti-CD31 (Santa Cruz Biotechnologies; at a dilution of 1:500), and anti-Ki-67 (Ventana Medical Systems, Inc.; AZ, United States) (ready to use). ZytoChem Plus (HRP) Polymer anti-Rabbit (Zytomed Systems, Berlin, Germany) (ready to use) and rabbit anti-goat immunoglobulin-HRP (Dako; CA, United States), were used for the detection of primary antibodies. The immunoreaction was visualized

Table 1 The primer sequences used in this study

Genes	Primer sequence
β-actin	F-5′ACCAACTGGGACGACATGGAGAA-3′
	R-5′GTGGTGGTGAAGCTGTAGCC-3′
IFN-α	F-5′GCTTTACTGATGGTCCTGGTGGTG-3′
	R-5′GAGATTCTGCTCATTTGTGCCAG-3′
IFN-β	F-5′GAATGGGAGGCTTGAATACTGCCT-3′
	R-5′TAGCAAAGATGTTCTGGAGCATCTC-3′
TNF-α	F-5′CTTCTCCTTCCTGATCGTGG-3′
	R-5′GCTGGTTATCTCTCAGCTCCA-3′
HBx	F-5′CACCTCTCTTTACGCGGACT-3′
	R-5′GGTCGTTGACATTGCAGAGA-3′
HBV PreS1	F-5′GGGTCACCATATTCTTGGGAAC-3′
	R-5′CCTGAGCCTGAGGGCTCCAC-3′

IFN: Interferon; TNF: Tumour necrosis factor; HBV: Hepatitis B virus; HBx: Hepatitis B virus X gene-encoded protein.

with ultraView Universal DAB Detection Kit (Ventana Medical Systems, Inc.). The nuclei were counterstained with Mayer's haematoxylin. Immunoreactions were measured in five microscopic fields per sample with 20 × objective magnification (Nikon Eclipse50i, Japan). The percentage of Ki67 was analysed by the ImmunoRatio web application[28].

Tumour vasculature imaging

Tumour vasculature imaging was performed as previously described[29]. Briefly, mice were anesthetised with an intraperitoneal injection of sodium pentobarbital (50 mg/kg BW). A catheter was inserted into the jugular vein for the application of fluorescence tracers. Then, the dorsal skin-fold chamber was removed, and the skin area around the chamber was fixed with modelling wax on a plate. To visualise the vascular lumen, a bolus of 0.1 mL of 5% fluorescein isothiocyanate-labelled dextran (FITC-dextran) was injected into the jugular vein. The tumour vasculature was visualised under a confocal microscope.

Quantitative gene expression

Total RNA was extracted from cell culture or xenograft tumour tissues using the RNeasy Mini kit (Qiagen, Hilden, Germany). One microgram of RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA, United States). Quantitative PCR amplification was performed with SYBR green (Applied Biosystems) on the Applied Biosystems 7500 Real-Time PCR System for 40 cycles. Derivation of the 2-ddCT method was applied for the relative quantification of mRNA expression. Beta-actin was used as an endogenous control. The primers used in this study are shown in Table 1.

HBV viral DNA analysis

Genomic DNA was extracted from cell culture or xenograft tumour tissues using the QIAamp DNA Mini Kit (Qiagen). Fifty nanograms of DNA of all samples were amplified to determine preS1 HBV gene expression relative to a standard copy number of HBV. The quantitative PCR amplification was performed with SYBR green (Applied Biosystems) on the Applied Biosystems 7500 Real-Time PCR System.

Statistical analysis

One-way ANOVA and *t*-test were applied using Prism 5 software (GraphPad Software Inc., San Diego, CA, United States). The results are shown as the mean ± SD, and differences of $P < 0.05$ were accepted as the level of significance.

RESULTS

Suppression of DLL4 delays HCC tumour growth and reduces VEGF factor.

To investigate the role of DLL4 in HCC, we transfected a DLL4 suppression construct, shDLL4 to knockdown DLL4 expression or control pGFP-V-RS plasmids into the HepG2.2.15 cell line. Clones of transfected cells with stable knockdown of DLL4 were selected with puromycin by limiting dilution assay. Various clones were selected and DLL4 expression was determined by western blot and compared with the control (Figure 1A). Clones with the strongest DLL4 suppression were chosen for xenograft transplantation. At 18 d and 30 d after shDLL4 HepG2.2.15 transplantation, mice showed significantly reduced tumour growth (Figure 1B) for both tumour volume (n = 4-6, $P < 0.0001$) and tumour weight (n = 4-6, $P < 0.05$ at 18 d and $P < 0.01$ at 30 d) compared with mice transplanted with control HepG2.2.15 cells (Figure 1C and D).

We monitored the expression of DLL4 and cleaved Notch1 expression in tumours taken from xenograft mice. Interestingly, the expression of DLL4 recovered in one mouse (#5 Figure 1E) and cleaved Notch1 was not significantly different in the implanted HepG2.2.15 tumours with shDLL4 compared with controls (Figure 1E and F).

DLL4 is mainly expressed in vascular endothelium and is related to VEGF expression, which promotes tumour angiogenesis[30-32]. To examine whether DLL4-expressing HCC is associated with VEGF expression, we analysed VEGF expression from the tumour xenograft. Interestingly, at 18 d after transplantation, the expression of VEGF was significantly decreased in implanted HepG2.2.15 tumours with shDLL4 compared with the control tumour ($P < 0.001$) (Figure 1E and F). VEGFR2 and CD31, tumour vasculature markers, were also significantly reduced in shDLL4 HepG2.2.15 compared with control HepG2.2.15 ($P < 0.05$ and $P < 0.001$, respectively) (Figures 1E and F, 2A and B, respectively). CD31 expression, which indicates neovascularization, was also decreased consistent with the Ki67 expression.

Next, we analysed cell proliferation by Ki67 expression in tumour xenografts using immunohistochemistry. The suppression of DLL4 reduced the percentage of cells with Ki67 nuclear positivity as shown at day 18 post transplantation in Figure 2B and 2D ($P < 0.05$). The tumour

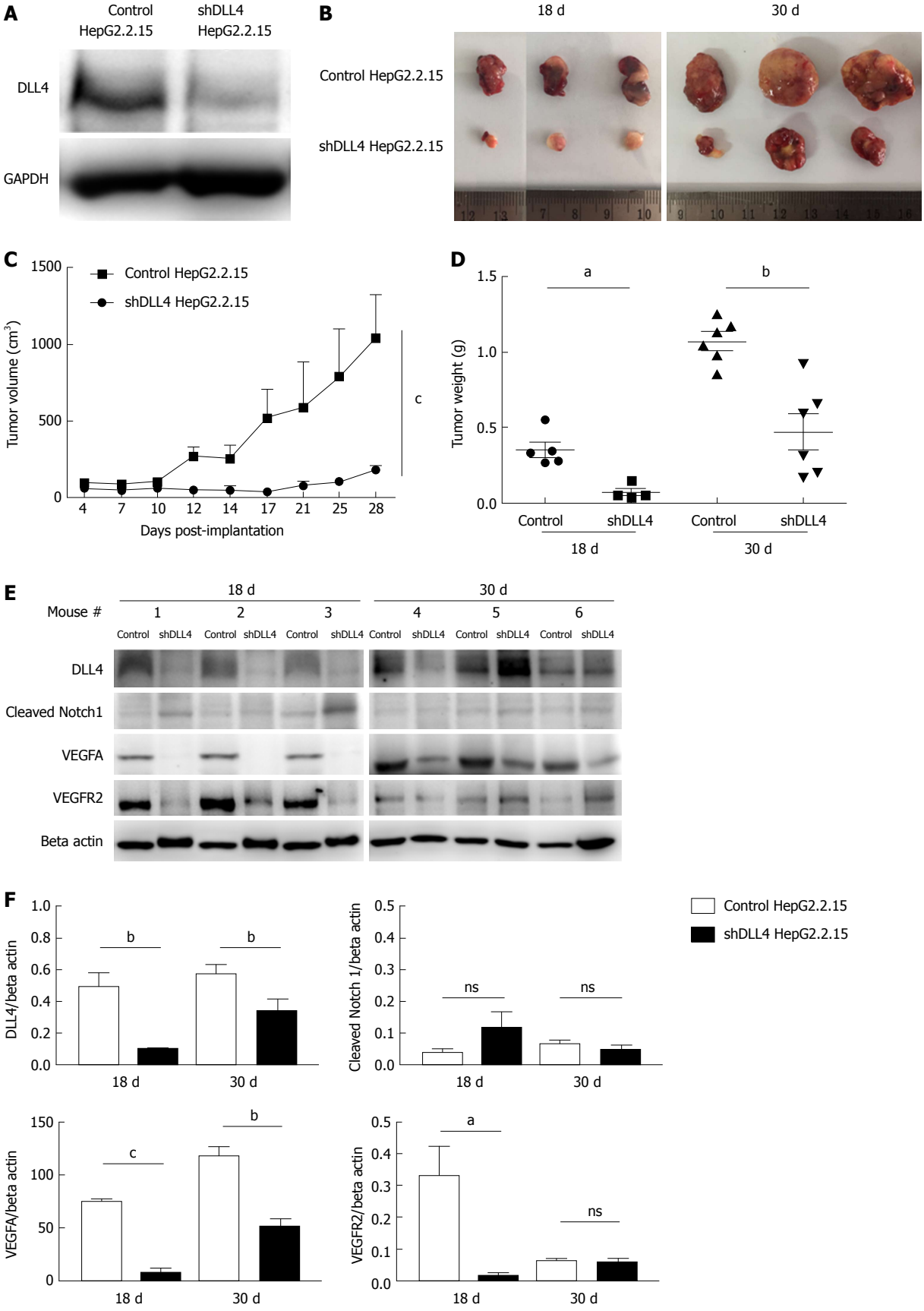

Figure 1 Delta-like ligand 4 expression promotes hepatitis B virus-associated hepatocellular carcinoma tumour growth *in vivo*. A: Western blot analyses of DLL4 expression in HepG2.2.15 stably transfected with shDLL4 or control vector. GAPDH was used as the loading control. B-D: HepG2.2.15 transfected with shDLL4 or control vector were subcutaneously injected into athymic nude mice (B) (1×10^7 cells per mouse, n = 4-6). Tumour volume (C) and tumour weights (D) are shown. At 18 d and 30 d after implantation, tumours were collected and analysed for DLL4, cleaved Notch1, VEGFA, and VEGFR2 by western blot. Beta-actin was used for the loading control. The blots cropped from different parts of the same gel (E). Band intensities from (E) were measured and the results are presented as the mean ± SD of three independent experiments (F). $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$.

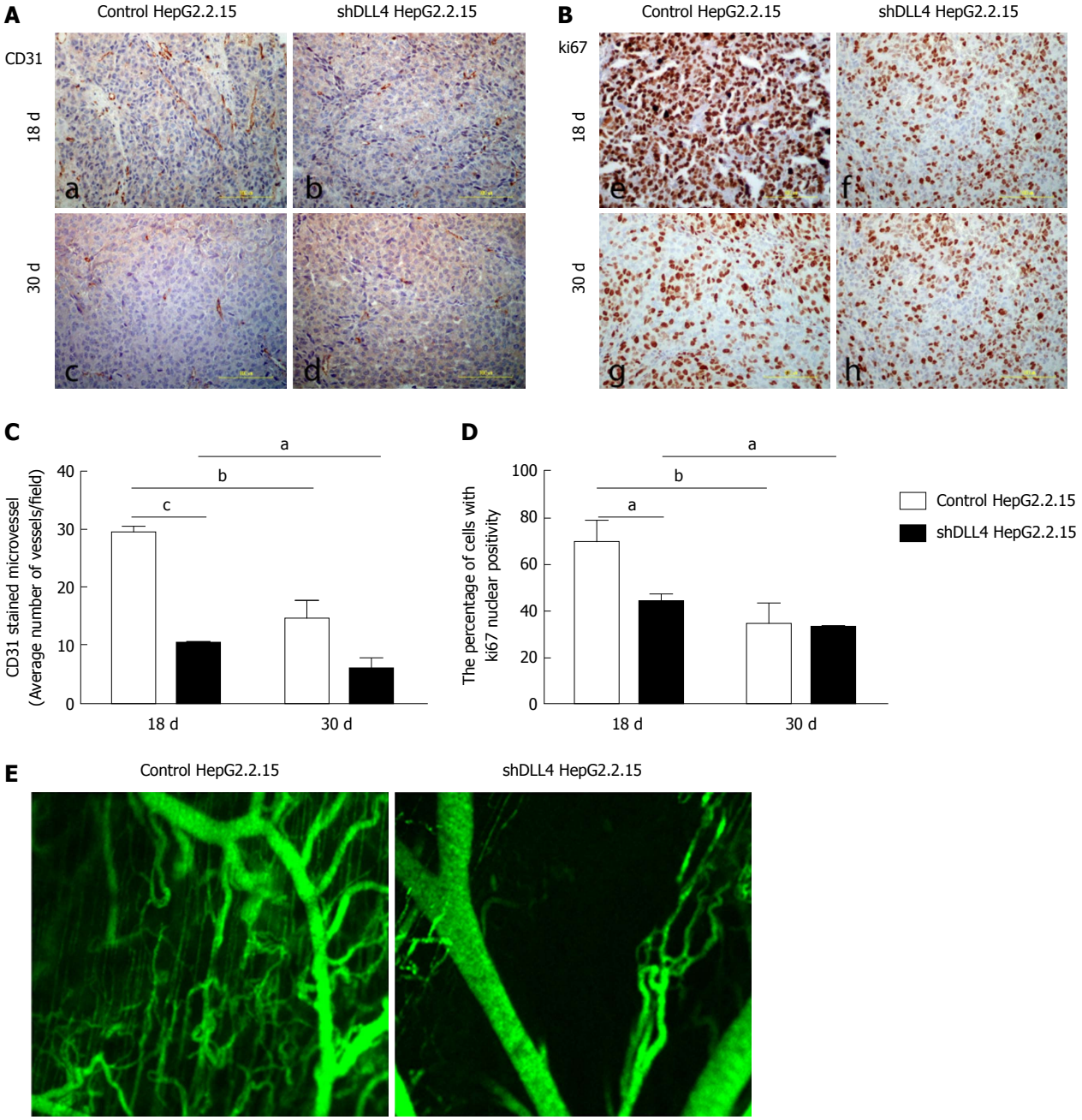

Figure 2 Suppression of Delta-like ligand 4 reduces tumour proliferation and neovasculature at the initiation stage of implantation. A and B: Immunohistochemical staining of CD31 (a-d) and Ki67 (e-h) shows mouse neovessels and tumour cell proliferation in paraffin sections of tumour xenografts, respectively. Five fields of each section were quantified for the amount of CD31 staining (C), and the percentage of Ki67 positive cells (D) in tumours transfected with shDLL4 or control vector at 18 d and 30 d after implantation. The tumour vasculature was measured after tumour implantation with shDLL4 HepG2.2.15 or control cells at 30 d (E). The data represent the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

vasculature was also decreased in shDLL4 HepG2.2.15 compared with control HepG2.2.15 (Figure 2E).

Interestingly, at 30 d after transplantation, the expression of VEGFR2, CD31, and Ki67 were not significantly different between shDLL4 HepG2.2.15 and control tumours. These data suggested that DLL4 may have an important role at the initiation stage of tumour proliferation.

Suppression of DLL4 increases HBV viral production in vivo

We have previously shown that *in vitro* HBV activated Notch signalling by increasing DLL4 had no effect on HBV viral replication[22]. To confirm our observation *in vivo*, we monitored viral production in the tumour xenograft. Unexpectedly, we found that HBV viral DNA and HBx mRNA expression were significantly increased in shDLL4 HepG2.2.15 compared with control HepG2.2.15 ($P < 0.05$ and $P < 0.01$, respectively). HBV preS1 protein was also increased in shDLL4 HepG2.2.15 at 18 d and 30 d after implantation (Figure 3A-C). We therefore measured the amount of type I interferon and found no difference in the level of IFN-α, IFN-β, or TNF-α (Figure 3D-F). Taken together, we found that a decrease in DLL4 expression in

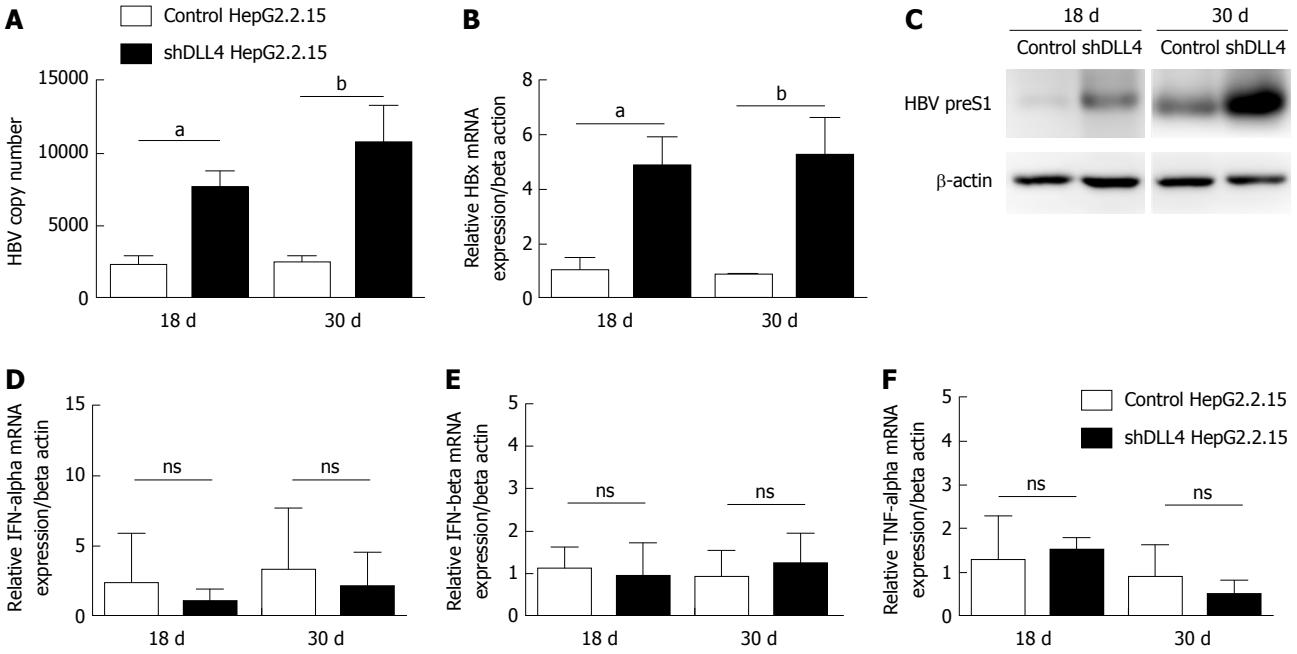

Figure 3 Suppression of Delta-like ligand 4 enhances hepatitis B virus viral replication (A), HBx mRNA expression (B), and HBs protein expression (C) *in vivo*. DNA, RNA, and proteins were extracted from tumour xenografts to analyse HBV viral components at 18 d and 30 d after implantation. The mRNA levels of human IFN-alpha (D), IFN-beta (E), and TNF-alpha (F) from tumours transfected with shDLL4 or control vectors were measured by quantitative RT-PCR and normalized to beta-actin mRNA expression. The data are represented by the mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$.

the HBV expressing HCC cell line *in vivo* reduced tumour cell proliferation and increased viral replication.

DISCUSSION

In this study, we followed up on our previous observation that HBx induced DLL4 in the HCC cell line and regulated cell survival at least *via* the activation of Notch1[22]. The effects of DLL4 suppression in an HCC cell line was observed at two main levels: (1) was the effect on tumour growth and (2) was the effect on viral replication. As expected, HepG2.2.15 with reduced DLL4 expression grew poorly in immunocompromised nude mice, compared with the siRNA transfected control. The suppression effect of *DLL4* remained intact 18 d after implantation but it was diminished in some, but not all mice, after 30 d. Interestingly, the level of cleaved Notch1 was not reduced in all tumours even when *DLL4* was successfully suppressed. Similarly, the level of *Hes1* and *Hey1*, two well-characterized Notch target genes, were also not affected by *DLL4* suppression in tumours (data not shown). It is possible that other Notch receptors besides Notch1 and Notch ligands, such as JAG1 or DLL3, are activated in the tumours. Indeed, HBx expression induced JAG1 expression in the HCC cell line[33]. In one study, suppressing both DLL4 and Jagged1 increased the inhibitory effect on the proliferation and invasiveness of human gastric carcinoma[34]. Although we cannot rule out the possibility that Notch receptors/ligands are activated in the tumour, our observation clearly showed that DLL4 is important for the tumour growth of the implanted HepG2.2.15 cell line. However, there are studies that suggested that a high level of DLL4 is associated with inhibition of tumour growth and metastasis in HCC[35,36]. Notch receptors have been suggested to play a role in both oncogenes and tumour-suppressor genes in different cell types[37,38]. We hypothesized that DLL4 may act as an oncogene in the initiation stage of tumour development, and then act as a tumour suppressor in the late stage depending on the DLL4 isoform or other tumour microenvironments. However, the dual function of DLL4 as a tumour-suppressor and oncogene needs to be further clarified.

The most striking effect of DLL4 knockdown on HepG2.2.15 *in vivo* was the reduction of angiogenesis factors, VEGFA and VEGFR2 (Figure 1E). In our study, we detected VEGFA of tumour (human) origin and VEGFR2 of host (mouse) origin. When the tumour vasculature was visualized, a reduced vasculature was observed in DLL4 knockdown tumours, consistent with the reduced expression of angiogenesis factors. CD31, an endothelial cell marker, was also reduced. DLL4 was reported to be involved in tumour angiogenesis[39]. Suppressing DLL4 in tumours resulted in non-productive angiogenesis and the suppression of tumour growth[40]. Our observation is in line with these reports and confirmed the importance of DLL4 in angiogenesis during tumour growth. DLL4 on tumour cells interacts with Notch receptors on host stromal/endothelial cells and helps tumour angiogenesis, thus improving tumour vascular function[41]. This event leads to increased tumour growth in some, but not all, types of cancer cells such as glioblastoma and prostate cancer. Our result is consistent with this observation, and we have added HCC as another tumour cell that

relies on DLL4 for vasculature formation. One intriguing observation from our study is that tumour (human) derived VEGF induces vasculature in the host (mouse).

Recent comprehensive and integrative genomic characterisation of hepatocellular carcinoma was performed on various HCC of different aetiologies. Although Notch receptors/ligands and the associated signalling molecules did not stand out as the prime mutated genes, the core Notch signalling was one of the pathways in HCC with enriched frequencies of functionally impactful mutations (ranked as No. 71)[42]. In HCV-related HCC, the Notch tumour signature genes (activation and deregulation) were found in 31.8% of patients (n = 91), suggesting a partial role of Notch signalling in promoting HCC in HCV infection[16]. This analysis highlighted the fact that Notch signalling may be involved in certain, but not all, subsets of HCC. In addition, it is not known whether HCC arising from other non-viral infections causes, but DLL4 has been linked to liver fibrosis and non-alcoholic steatohepatitis pathogenesis[43].

Tumour cell proliferation was significantly reduced when *DLL4* was suppressed. This effect was determined by the reduction in Ki67, which was robust at an early stage (18 d post transplantation). There are two likely scenarios for the effect of DLL4 on tumour cell growth. One possibility is that reducing DLL4 expression decreased Notch signalling and as a result, cell growth *in vivo* was compromised. Many studies have reported the cell proliferation promoting effect of Notch in HCC[44-46]. Suppression of *DLL4* in HepG2.2.15 reduced cell viability and interfered with cell cycle progression *in vitro*[22]. Another possibility is that the defect in angiogenesis within tumours played a key role in reducing cell proliferation. This effect together with reduced angiogenesis may contribute to severe growth retardation *in vivo*.

Unexpectedly, increased viral replication was observed in HepG2.2.15 upon DLL4 suppression *in vivo*. We previously reported that suppressing *DLL4* in HepG2.2.15 *in vitro* did not alter viral replication[22]. This discrepancy highlights the more complex multi-cellular interactions *in vivo*. It is unclear how DLL4 suppression promoted HBV viral replication. However, there may be two possibilities: the extrinsic and/or the intrinsic effect. If suppressing DLL4 created an environment that was friendly to viral replication, such as by inducing less anti-viral cytokines IFNα/β or promoting skewed helper T cell polarization, then this is considered an extrinsic effect. In contrast, what we observed is that there was no significant difference in IFNα/β level between control and tumour with suppressed DLL4 and the mice lacked adaptive immune response. Thus, we concluded that the viral replication promoting effect must be intrinsic. Namely the intracellular environment with reduced DLL4 levels allowed the virus to replicate better. Currently, there is no evidence that this effect is dependent upon reduced Notch signalling.

There are several reports on the effect of viral infection and Notch ligand expression. In Dengue virus infection, DLL1 and DLL4 were upregulated in antigen presenting cells *via* the IFN-β signalling pathway, which in turn influenced helper T cell responses[47]. Respiratory syncytial virus also induced DLL4 expression in dendritic cells to direct helper T cell polarization[48]. In another report, Kaposi sarcoma herpesvirus induced the expression of DLL4 and JAG1 to alter cell cycle regulating genes in neighbouring cells[49]. However, there has been no report on the impact of DLL4 expression on HBV replication. Our observation that suppressing DLL4 decreased angiogenesis indicates it might induce hypoxic conditions within the tumour. Indeed, various studies have linked the expression of hypoxia-inducible factor and viral replication during carcinogenesis[50,51]. If hypoxia and cellular stress due to defective angiogenesis caused by DLL4 suppression is the cause for enhanced viral replication, then it is speculated that suppressing DLL4 expression may promote viral replication in other cell types as well. Whether increased HBV replication is the cause for reduced tumour growth is not determined.

Taken together, we report the novel findings that DLL4 in an HBV expressing HCC cell line regulated tumour growth, angiogenesis, and viral replication in a mouse model of xenograft transplantation. Therefore, DLL4 may be a good candidate for HCC therapy.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) has been studied for many decades. However, the molecular mechanism is still unclear. Notch signaling in HCC pathogenesis is controversial, but we found that HBx promoted HBV-associated HCC proliferation through Delta-like ligand 4 (DLL4) (Notch ligand) in an *in vitro* study. However, the effect of DLL4 inhibition in HCC has not been explored.

Research motivation

DLL4 has a potential function for angiogenesis that supports tumour growth. The understanding of DLL4 mechanism might lead to identifying a new target for HCC therapy.

Research objective

We investigated the role of DLL4 on tumour growth in HCC associated with HBV in a xenograft model and detailed the molecular mechanism of HCC.

Research methods

We inhibited the DLL4 expression in HBV-associated HCC, and then subcutaneously implanted in nude mice. We analysed the ability for tumour growth, angiogenesis regulators (VEGF-A, VEGF-R2) expression, neovasculature, and HBV expression in tumour xenografts.

Research results

The tumour volume, VEGF-A, and VEGF-R2 were significantly decreased in mice implanted with suppressed DLL4 HCC compared with the control group. The suppression of DLL4 expression in tumour cells reduced cell proliferation and the formation of new blood vessels in tumours. Unexpectedly, viral replication increased in DLL4 suppressed tumours.

Research conclusions

This study demonstrates that DLL4 is important in regulating the tumour growth and neovascularization in HBV-associated HCC, as well as suppressing HBV replication *in vivo*.

Research perspective

This study showed that DLL4 is essential for the tumour growth of the implanted HBV-associated HCC cell line, especially in the initiation stage of tumour growth. However, the role of DLL4 as a tumour oncogene and tumour suppressor gene in HCC needs to further clarification.

REFERENCES

1 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]

2 **El-Serag HB**. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; **142**: 1264-1273.e1 [PMID: 22537432 DOI: 10.1053/j.gastro.2011.12.061]

3 **Yang HI**, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ; Taiwan Community-Based Cancer Screening Project Group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med* 2002; **347**: 168-174 [PMID: 12124405 DOI: 10.1056/NEJMoa013215]

4 **Benhenda S**, Cougot D, Buendia MA, Neuveut C. Hepatitis B virus X protein molecular functions and its role in virus life cycle and pathogenesis. *Adv Cancer Res* 2009; **103**: 75-109 [PMID: 19854353 DOI: 10.1016/S0065-230X(09)03004-8]

5 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

6 **Hori K**, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. *J Cell Sci* 2013; **126**: 2135-2140 [PMID: 23729744 DOI: 10.1242/jcs.127308]

7 **Lai EC**. Notch signaling: control of cell communication and cell fate. *Development* 2004; **131**: 965-973 [PMID: 14973298 DOI: 10.1242/dev.01074]

8 **Yamamoto S**, Schulze KL, Bellen HJ. Introduction to Notch signaling. *Methods Mol Biol* 2014; **1187**: 1-14 [PMID: 25053477 DOI: 10.1007/978-1-4939-1139-4_1]

9 **Koch U**, Radtke F. Notch and cancer: a double-edged sword. *Cell Mol Life Sci* 2007; **64**: 2746-2762 [PMID: 17687513 DOI: 10.1007/s00018-007-7164-1]

10 **South AP**, Cho RJ, Aster JC. The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol* 2012; **23**: 458-464 [PMID: 22309843 DOI: 10.1016/j.semcdb.2012.01.017]

11 **Timmerman LA**, Grego-Bessa J, Raya A, Bertrán E, Pérez-Pomares JM, Díez J, Aranda S, Palomo S, McCormick F, Izpisúa-Belmonte JC, de la Pompa JL. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev* 2004; **18**: 99-115 [PMID: 14701881 DOI: 10.1101/gad.276304]

12 **Gao J**, Dong Y, Zhang B, Xiong Y, Xu W, Cheng Y, Dai M, Yu Z, Xu H, Zheng G. Notch1 activation contributes to tumor cell growth and proliferation in human hepatocellular carcinoma HepG2 and SMMC7721 cells. *Int J Oncol* 2012; **41**: 1773-1781 [PMID: 22922832 DOI: 10.3892/ijo.2012.1606]

13 **Gramantieri L**, Giovannini C, Lanzi A, Chieco P, Ravaioli M, Venturi A, Grazi GL, Bolondi L. Aberrant Notch3 and Notch4 expression in human hepatocellular carcinoma. *Liver Int* 2007; **27**: 997-1007 [PMID: 17696940 DOI: 10.1111/j.1478-3231.2007.01544.x]

14 **Hu L**, Xue F, Shao M, Deng A, Wei G. Aberrant expression of Notch3 predicts poor survival for hepatocellular carcinomas. *Biosci Trends* 2013; **7**: 152-156 [PMID: 23836039]

15 **Ning L**, Wentworth L, Chen H, Weber SM. Down-regulation of Notch1 signaling inhibits tumor growth in human hepatocellular carcinoma. *Am J Transl Res* 2009; **1**: 358-366 [PMID: 19956448]

16 **Villanueva A**, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, Rodriguez-Carunchio L, Solé M, Thung S, Stanger BZ, Llovet JM. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. *Gastroenterology* 2012; **143**: 1660-1669.e7 [PMID: 22974708 DOI: 10.1053/j.gastro.2012.09.002]

17 **Qi R**, An H, Yu Y, Zhang M, Liu S, Xu H, Guo Z, Cheng T, Cao X. Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. *Cancer Res* 2003; **63**: 8323-8329 [PMID: 14678992]

18 **Viatour P**, Ehmer U, Saddic LA, Dorrell C, Andersen JB, Lin C, Zmoos AF, Mazur PK, Schaffer BE, Ostermeier A, Vogel H, Sylvester KG, Thorgeirsson SS, Grompe M, Sage J. Notch signaling inhibits hepatocellular carcinoma following inactivation of the RB pathway. *J Exp Med* 2011; **208**: 1963-1976 [PMID: 21875955 DOI: 10.1084/jem.20110198]

19 **Wang C**, Qi R, Li N, Wang Z, An H, Zhang Q, Yu Y, Cao X. Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human hepatocellular carcinoma cells by inhibiting Akt/Hdm2-mediated p53 degradation and up-regulating p53-dependent DR5 expression. *J Biol Chem* 2009; **284**: 16183-16190 [PMID: 19376776 DOI: 10.1074/jbc.M109.002105]

20 **Sun Q**, Wang R, Luo J, Wang P, Xiong S, Liu M, Cheng B. Notch1 promotes hepatitis B virus X protein-induced hepatocarcinogenesis *via* Wnt/β-catenin pathway. *Int J Oncol* 2014; **45**: 1638-1648 [PMID: 25017705 DOI: 10.3892/ijo.2014.2537]

21 **Wang F**, Zhou H, Yang Y, Xia X, Sun Q, Luo J, Cheng B. Hepatitis B virus X protein promotes the growth of hepatocellular carcinoma by modulation of the Notch signaling pathway. *Oncol Rep* 2012; **27**: 1170-1176 [PMID: 22218807 DOI: 10.3892/or.2012.1620]

22 **Kongkavitoon P**, Tangkijvanich P, Hirankarn N, Palaga T. Hepatitis B Virus HBx Activates Notch Signaling *via* Delta-Like 4/Notch1 in Hepatocellular Carcinoma. *PLoS One* 2016; **11**: e0146696 [PMID: 26766040 DOI: 10.1371/journal.pone.0146696]

23 **Hellström M**, Phng LK, Gerhardt H. VEGF and Notch signaling: the yin and yang of angiogenic sprouting. *Cell Adh Migr* 2007; **1**: 133-136 [PMID: 19262131 DOI: 10.4161/cam.1.3.4978]

24 **Patel NS**, Li JL, Generali D, Poulsom R, Cranston DW, Harris AL. Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res* 2005; **65**: 8690-8697 [PMID: 16204037 DOI: 10.1158/0008-5472.CAN-05-1208]

25 **Ridgway J**, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman I, Singh M, Chien M, Tan C, Hongo JA, de Sauvage F, Plowman G, Yan M. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 2006; **444**: 1083-1087 [PMID: 17183323 DOI: 10.1038/nature05313]

26 **Chiorean EG**, LoRusso P, Strother RM, Diamond JR, Younger A, Messersmith WA, Adriaens L, Liu L, Kao RJ, DiCioccio AT, Kostic A, Leek R, Harris A, Jimeno A. A Phase I First-in-Human Study of Enoticumab (REGN421), a Fully Human Delta-like Ligand 4 (Dll4) Monoclonal Antibody in Patients with Advanced Solid Tumors. *Clin Cancer Res* 2015; **21**: 2695-2703 [PMID: 25724527 DOI: 10.1158/1078-0432.CCR-14-2797]

27 **Smith DC**, Eisenberg PD, Manikhas G, Chugh R, Gubens MA, Stagg RJ, Kapoun AM, Xu L, Dupont J, Sikic B. A phase I dose escalation and expansion study of the anticancer stem cell agent demcizumab (anti-DLL4) in patients with previously treated solid tumors. *Clin Cancer Res* 2014; **20**: 6295-6303 [PMID: 25324140 DOI: 10.1158/1078-0432.CCR-14-1373]

28 **Tuominen VJ**, Ruotoistenmäki S, Viitanen A, Jumppanen M, Isola J. ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67. *Breast Cancer Res* 2010; **12**: R56 [PMID: 20663194 DOI: 10.1186/bcr2615]

29 **Patumraj S**, Yoysungnoen P, Kachonrattanadet P, Wirachwong P. Tumor neocapillary density in hepatocellular carcinoma cells implanted nude mice model. *Clin Hemorheol Microcirc* 2005; **33**: 137-144 [PMID: 16151261]

30 **Miles KM**, Seshadri M, Ciamporcero E, Adelaiye R, Gillard B, Sotomayor P, Attwood K, Shen L, Conroy D, Kuhnert F, Lalani AS, Thurston G, Pili R. Dll4 blockade potentiates the anti-tumor effects of VEGF inhibition in renal cell carcinoma patient-derived xenografts.

PLoS One 2014; **9**: e112371 [PMID: 25393540 DOI: 10.1371/journal.pone.0112371]

31 **Vincent F**, Bonnin P, Clemessy M, Contrerès JO, Lamandé N, Gasc JM, Vilar J, Hainaud P, Tobelem G, Corvol P, Dupuy E. Angiotensinogen delays angiogenesis and tumor growth of hepatocarcinoma in transgenic mice. *Cancer Res* 2009; **69**: 2853-2860 [PMID: 19318581 DOI: 10.1158/0008-5472.CAN-08-2484]

32 **Xiong YQ**, Sun HC, Zhang W, Zhu XD, Zhuang PY, Zhang JB, Wang L, Wu WZ, Qin LX, Tang ZY. Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. *Clin Cancer Res* 2009; **15**: 4838-4846 [PMID: 19638466 DOI: 10.1158/1078-0432.CCR-08-2780]

33 **Gao J**, Chen C, Hong L, Wang J, Du Y, Song J, Shao X, Zhang J, Han H, Liu J, Fan D. Expression of Jagged1 and its association with hepatitis B virus X protein in hepatocellular carcinoma. *Biochem Biophys Res Commun* 2007; **356**: 341-347 [PMID: 17359939 DOI: 10.1016/j.bbrc.2007.02.130]

34 **Sun HW**, Wu C, Tan HY, Wang QS. Combination DLL4 with Jagged1-siRNA can enhance inhibition of the proliferation and invasiveness activity of human gastric carcinoma by Notch1/VEGF pathway. *Hepatogastroenterology* 2012; **59**: 924-929 [PMID: 22020917 DOI: 10.5754/hge11484]

35 **Chen H**, Yang L, Zang S, Zhuo L, Fang X, Zhang Y, Li K, Song K, A H. High level of Delta-like ligand 4 suppresses the metastasis of hepatocellular carcinoma. *Int J Clin Exp Pathol* 2016; **9**: 2989-2997

36 **Liu X**, Zhou J, Zhou N, Zhu J, Feng Y, Miao X. SYNJ2BP inhibits tumor growth and metastasis by activating DLL4 pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res* 2016; **35**: 115 [PMID: 27440153 DOI: 10.1186/s13046-016-0385-0]

37 **Lobry C**, Oh P, Mansour MR, Look AT, Aifantis I. Notch signaling: switching an oncogene to a tumor suppressor. *Blood* 2014; **123**: 2451-2459 [PMID: 24608975 DOI: 10.1182/blood-2013-08-355818]

38 **Aster JC**, Pear WS, Blacklow SC. The Varied Roles of Notch in Cancer. *Annu Rev Pathol* 2017; **12**: 245-275 [PMID: 27959635 DOI: 10.1146/annurev-pathol-052016-100127]

39 **Li JL**, Harris AL. Crosstalk of VEGF and Notch pathways in tumour angiogenesis: therapeutic implications. *Front Biosci (Landmark Ed)* 2009; **14**: 3094-3110 [PMID: 19273260 DOI: 10.2741/3438]

40 **Noguera-Troise I**, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 2006; **444**: 1032-1037 [PMID: 17183313 DOI: 10.1038/nature05355]

41 **Li JL**, Sainson RC, Shi W, Leek R, Harrington LS, Preusser M, Biswas S, Turley H, Heikamp E, Hainfellner JA, Harris AL. Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo. *Cancer Res* 2007; **67**: 11244-11253 [PMID: 18056450 DOI: 10.1158/0008-5472.CAN-07-0969]

42 **Cancer Genome Atlas Research Network.** Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

43 **Kawaguchi K**, Honda M, Kaneko S. The Role of Notch Signaling in Liver Diseases: Contribution to Development and Cancer. *Int J Cancer Clin Res* 2017; **4**: 079 [DOI: 10.23937/2378-3419/1410079]

44 **Lu J**, Xia Y, Chen K, Zheng Y, Wang J, Lu W, Yin Q, Wang F, Zhou Y, Guo C. Oncogenic role of the Notch pathway in primary liver cancer. *Oncol Lett* 2016; **12**: 3-10 [PMID: 27347091 DOI: 10.3892/ol.2016.4609]

45 **Strazzabosco M**, Fabris L. Notch signaling in hepatocellular carcinoma: guilty in association! *Gastroenterology* 2012; **143**: 1430-1434 [PMID: 23099244 DOI: 10.1053/j.gastro.2012.10.025]

46 **Zhu B**, Sun L, Luo W, Li M, Coy DH, Yu L, Yu W. Activated Notch signaling augments cell growth in hepatocellular carcinoma *via* up-regulating the nuclear receptor NR4A2. *Oncotarget* 2017; **8**: 23289-23302 [PMID: 28423575 DOI: 10.18632/oncotarget.15576]

47 **Li Y**, Wu S, Pu J, Huang X, Zhang P. Dengue virus up-regulates expression of notch ligands Dll1 and Dll4 through interferon-β signalling pathway. *Immunology* 2015; **144**: 127-138 [PMID: 25041739 DOI: 10.1111/imm.12357]

48 **Schaller MA**, Neupane R, Rudd BD, Kunkel SL, Kallal LE, Lincoln P, Lowe JB, Man Y, Lukacs NW. Notch ligand Delta-like 4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines. *J Exp Med* 2007; **204**: 2925-2934 [PMID: 17998388 DOI: 10.1084/jem.20070661]

49 **Emuss V**, Lagos D, Pizzey A, Gratrix F, Henderson SR, Boshoff C. KSHV manipulates Notch signaling by DLL4 and JAG1 to alter cell cycle genes in lymphatic endothelia. *PLoS Pathog* 2009; **5**: e1000616 [PMID: 19816565 DOI: 10.1371/journal.ppat.1000616]

50 **Cuninghame S**, Jackson R, Zehbe I. Hypoxia-inducible factor 1 and its role in viral carcinogenesis. *Virology* 2014; **456-457**: 370-383 [PMID: 24698149 DOI: 10.1016/j.virol.2014.02.027]

51 **Wilson GK**, Tennant DA, McKeating JA. Hypoxia inducible factors in liver disease and hepatocellular carcinoma: current understanding and future directions. *J Hepatol* 2014; **61**: 1397-1406 [PMID: 25157983 DOI: 10.1016/j.jhep.2014.08.025]

P- Reviewer: Abd-Elsalam A, Abid S, Ahn KS, Li W
S- Editor: Gong ZM **L- Editor**: Filipodia **E- Editor**: Yin SY

Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
http://www.wjgnet.com

ISSN 1007-9327