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

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 (0000-0002-9296-8671); 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.

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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

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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

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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.

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-tumorigenic 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 °C in a 5% CO₂ humidified incubator.

Generation of stable DLL4 knockdown cell lines
HepG2.2.15 cells were 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 per 1 μg of shRNA vector into 1 × 10⁶ cells per well in a 12-well plate. After 48 h, DLL4 mRNA expression was determined in transfected cells. The highest efficacy of shRNA vector (5′-ACCAGAAGAAGGAGCTGGAAGTGGACTGT-3′) 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 transplanted at a concentration of 1 × 10⁶ cells/mL. One milliliter 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 (cm³) was measured every three days until 18 d and 30 d using Vernier calipers and calculated using the formula: (length × width²)/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 °C for 20 min). The tissue sections were treated with 3% H₂O₂ for 15 min and blocked with normal serum for 30 min, then incubated with primary antibody in a humidity chamber at 4 °C 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). ZyroChem Plus (HRP) Polymer anti-Rabbit (Zytomedi 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′ACCAACTGGAGCATGGAGGA-3′ R-5′CTGTTGAGCAAGCTGTCGCC-3′                          |
| IFN-α     | F-5′GCGTTCTGATGCTGACAGCTG-3′ R-5′GTGCTGAGCAAGCTGTCGCC-3′                          |
| IFN-β     | F-5′GCACTGAGGAGCTGTCGCC-3′ R-5′TTAGCTGACAGCTGTCGCC-3′                           |
| TNF-α     | F-5′GCACTGAGGAGCTGTCGCC-3′ R-5′TTAGCTGACAGCTGTCGCC-3′                           |
| HBx       | F-5′GCTGGTTATCTCTCAGCTCCA-3′ R-5′GCTGGTTATCTCTCAGCTCCA-3′                      |
| HBV PreS1 | F-5′GGTGCACCATATTCTCAGGAAC-3′ R-5′GCTGGTTATCTCTCAGCTCCA-3′                      |

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.
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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). *P < 0.05; †P < 0.01; ‡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-\(\alpha\), IFN-\(\gamma\), or TNF-\(\alpha\) (Figure 3D-F). Taken together, we found that a decrease in DLL4 expression in
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[32]. 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 was observed at two main levels: (1) was the effect on viral replication and (2) was the effect on viral replication. HBx expression/beta actin mRNA expression. The data are represented by the mean ± SD. *P < 0.05; **P < 0.01.

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.

Suppression of DLL4 in angiogenesis during tumour growth. DLL4 was reported to be involved in tumour angiogenesis[38]. DLL4 promotes 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...
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reduced cell viability and interfered with cell cycle progression and Notch ligand expression. In Dengue virus reduced Notch signalling. There is no evidence that this effect is dependent upon levels allowed the virus to replicate better. Currently, the viral replication promoting effect must be intrinsic.

In HepG2.2.15 in vivo upon DLL4 suppression in vitro HBV expression in tumour xenografts. We analysed the ability for 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 HBV promoted HBV-associated HCC proliferation through Delta-like ligand 4 (DLL4) 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, neovascularization, 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.
tumour growth. However, the role of DLL4 as a tumour oncogene and tumour suppressor gene in HCC needs to further clarification.

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