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***Basic Study***

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

Kunanopparat A *et al.* DLL4 promotes tumour growth

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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 silenced *DLL4* expression in an HBV genome harbouring 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 xenograft was 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 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 silenced *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, in DLL4 silencing tumours, increased viral replication was observed.

***CONCLUSION***

This study demonstrates that DLL4 is important in regulating the tumour growth of HBV-associated HCC, accompanied with neovascularization and intrinsically suppresses HBV replication.

**Key words:** Notch signalling; Delta-like ligand 4; hepatocellular carcinoma; 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, was significantly decreased in HCC xenograft tumours with silencing of 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 findings that DLL4 in HBV-genome harboring HCC cell line plays a key role in regulating tumour growth, angiogenesis and viral replication in a mouse model of xenograft transplantation.

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**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](#_ENREF_1)].Hepatitis B virus infection is the most prevalent cause of HCC in developing countries. Although a hepatitis B virus (HBV) vaccine has been 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](#_ENREF_2),[3](#_ENREF_3)]. The molecular mechanisms of HBV-associated HCC are poorly understood[[4](#_ENREF_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](#_ENREF_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](#_ENREF_6),[7](#_ENREF_7)]. In mammals, the Notch pathway comprises 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](#_ENREF_8)]. Dysregulation of Notch signalling has been reported in many types of cancer as either a tumour suppressor, or tumour promotor depending on the type of cancer[[9-11](#_ENREF_9)]. 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 the inhibition of Notch by a gamma secretase inhibitor or Notch target genes suppressed tumour growth[[12-16](#_ENREF_12)]. Several studies have also suggested that Notch is a tumour suppressor in HCC[[15](#_ENREF_15),[17-19](#_ENREF_17)]. However, more evidence supports the protumourigenic role of Notch in HCC carcinogenesis and progression, especially in HBV-associated HCC[[20](#_ENREF_20),[21](#_ENREF_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, a HBV-genome harbouring HCC cell line[[22](#_ENREF_22)].

Strong evidence indicates that DLL4 regulates angiogenesis and controls the balance of endothelial tip and stalk cell differentiation induced by VEGF[[23](#_ENREF_23)]. DLL4 is highly expressed in tumour endothelial cells for tumour angiogenesis, which is the primary signal for tumour progression[[24](#_ENREF_24)]. The inhibition of DLL4 in tumour endothelial cells suppressed tumour growth by inducing non-productive angiogenesis[[25](#_ENREF_25)]. Currently, a DLL4 neutralizing Ab has been developed and is being tested in a clinical trial for anticancer therapy in various cancers[[26](#_ENREF_26),[27](#_ENREF_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***

HBV-expressing HCC (HepG2.2.15) cell line and 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 silenced cell lines***

HepG2.2.15 cells line was transfected with a set of DLL4 shRNA (Origenes 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 × 105 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 silenced DLL4 were expended, and DLL4 expression was analysed by western blot analysis and compared with scramble control. The strongest silenced DLL4 clone was 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 scramble control HepG2.2.15 cells were trypsinized at a concentration of 1 × 107 cells/ml. One ml of the cells were 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 to 18 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 tumor 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 Ab (Santa Cruz Biotechnologies; at a dilution of 1:500), and anti-Ki-67 Ab (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 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](#_ENREF_28)].

***Tumour vasculature imaging***

Tumour vasculature imaging was performed as previously described[[29](#_ENREF_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.

***Statistics***

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**

***Silencing of DLL4 delays HCC tumour growth and reduces VEGF factor.***

To investigate the role of DLL4 in HCC, we transfected DLL4 silencing 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 DLL4 silencing were selected with puromycin by limiting dilution assay. Various clones were selected and DLL4 expression was determined by western blot and compared with the scramble control (Figure 1A). Clones with the strongest DLL4 silencing effect were chosen for xenograft transplantation. At 18 days 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 and *P* < 0.01, respectively) 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](#_ENREF_30)]. 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 implanted 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) (Figure 1E-F and 2A-B, respectively). CD31 expression, which indicates neovascularization, was also decreased consistent with Ki67 expression.

Next, we analysed cell proliferation by Ki67 expression in tumour xenografts using immunohistochemistry. The silencing of DLL4 reduced the percentage of cells with Ki67 nuclear positivity as shown at day 18 post transplantation in Figure 2B and D (*P* < 0.05). The tumour vasculature was also decreased in shDLL4 HepG2.2.15 compared with control HepG2.2.15 (Figure 2E).

Interestingly, at 30 days 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.

***Silencing 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](#_ENREF_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 days 18 and 30 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 the HBV-harbouring HCC cell line reduced argument factor, 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](#_ENREF_22)].The effects of DLL4 silencing in HCC cell line was observed at two main levels, one was the effect on tumour growth and the other was the effect on viral replication. As expected, HepG2.2.15 with reduced DLL4 expression grew poorly in immunocompromised nude mice, compared with the scramble siRNA transfected control. The silencing effect of *DLL4* remained intact after day 18 after implantation but it was diminished in some, but not all mice, at day 30. Interestingly, the level of cleaved Notch1 was not reduced in all tumours even when *DLL4* was successfully silenced. Similarly, the level of *Hes1* and *Hey1*, two well-characterized Notch target genes, were also not affected by *DLL4* silencing 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](#_ENREF_33)]. In one study, silencing both DLL4 and Jagged1 increased the inhibitory effect on the proliferation and invasiveness of human gastric carcinoma[[34](#_ENREF_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 were studies suggested that the high level of DLL4 associated with inhibition of tumour growth and metastasis in HCC[[35](#_ENREF_35),[36](#_ENREF_36)]. Notch receptors have been suggested to play a role in both oncogenes and tumour-suppressor gene in different cell types[[37](#_ENREF_37),[38](#_ENREF_38)]. We hypothesized that DLL4 may act as an oncogenic in the initiation stage of tumour development and then act as a tumour suppressor in the late stage for inhibiting tumour metastasis depend on the DLL4 isoform or other tumour microenvironments. However, the dual function of DLL4 as tumour-suppressor and oncogenicity associated their isoform need 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](#_ENREF_39)]. Blocking DLL4 in tumours results in non-productive angiogenesis and the suppression of tumour growth[[40](#_ENREF_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](#_ENREF_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 (murine).

Recent comprehensive and integrative genomic characterization of hepatocellular carcinoma was performed on various HCC of different etiologies. 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](#_ENREF_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](#_ENREF_16)]. This analysis result highlights 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 infection causes but DLL4 has been linked to liver fibrosis and non-alcoholic steatohepatitis pathogenesis[[43](#_ENREF_43)].

How does DLL4 regulate tumour growth? Tumour cell proliferation was significantly reduced when *DLL4* was silenced. This effect as determined by the reduction in Ki67, 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](#_ENREF_44)]. Silencing of *DLL4* in HepG2.2.15 reduced cell viability and interfered with cell cycle progression *in vitro*[[22](#_ENREF_22)]. Another possibility is that the defect in angiogenesis within tumours plays 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 silencing *in vivo*. We previously reported that silencing *DLL4* in HepG2.2.15 *in vitro* did not alter viral replication[[22](#_ENREF_22)]. This discrepancy highlights the more complex multi-cellular interactions *in vivo*. How does DLL4 silencing promote HBV viral replication? There are two possibilities, the intrinsic and/or the extrinsic effect. If silencing DLL4 creates environment that are friendly to viral replication such as inducing less anti-viral cytokines IFN/or promoting skewed helper T cell polarization, this is considered an extrinsic effect. In contrast, what we observed is that there was no significant difference in IFN/ level between control and tumour with silenced 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 reducing DLL4 level allows the virus to replicate better. Currently, there is no evidence that this effect is dependent upon reducing 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](#_ENREF_47)]. Respiratory syncytial virus also induced DLL4 expression in dendritic cells to direct helper T cell polarization[[48](#_ENREF_48)]. In another report, Kaposi sarcoma herpesvirus induced the expressions of DLL4 and JAG1 to alter cell cycle regulating genes in neighbouring cells[[49](#_ENREF_49)]. However, there has been no report on the impact of DLL4 expression on HBV replication. Our observation that reducing 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](#_ENREF_50),[51](#_ENREF_51)]. If hypoxia and cellular stress due to defective angiogenesis caused by DLL4 silencing is the cause for enhancing viral replication, it is speculated that silencing 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 determine.

Taken together, we report the novel findings that DLL4 in an HBV-genome harbouring HCC cell line regulates 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 explored 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 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 inhibit the DLL4 expression in HBV-associated HCC, and then subcutaneously implanted in nude mice. We analysed the ability of tumour growth, angiogenesis regulators (VEGF-A, VEGF-R2) expression, neovasculature, and HBV expression in tumour xenograft.

***Research results***

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

***Research conclusions***

This study demonstrates that DLL4 is important in regulating the tumour growth of HBV-associated HCC, accompanied with neovascularization and suppresses 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.

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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 × 107 cells per mouse, *n* = 4–6). Tumour volume (C) and tumour weights (D) are shown. At 18 days and 30 days 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). a*P* < 0.05; b*P* < 0.01; e*P* < 0.001.



**Figure 2 Silencing 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 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; e*P* < 0.001.



**Figure 3 Silencing 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 days 18 and 30 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.

**Table 1 The primer sequence 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.