

The Role of DLLs in Cancer: A Novel Therapeutic Target

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Abstract: Delta-like ligands (DLLs) control Notch signaling. DLL1, DLL3 and DLL4 are frequently deregulated in cancer and influence tumor growth, the tumor vasculature and tumor immunity, which play different roles in cancer progression. DLLs have attracted intense research interest as anti-cancer therapeutics. In this review, we discuss the role of DLLs in cancer and summarize the emerging DLL-relevant targeting methods to aid future studies.

Keywords: delta-like ligands, notch signaling, cancer, therapy

Introduction

Notch is an evolutionary-conserved signaling system that regulates cell fate through local cell–cell interactions. In mammals, four Notch receptors (Notch1–4) and five Notch ligands (Delta-like 1, 3, 4 and Jagged 1–2) are expressed (Figure 1A). Notch receptors are cleaved (S1 cleavage) in the Golgi apparatus and traffic to the cell surface as a transmembrane heterodimeric protein. When interacting with Notch ligands in adjacent cells, the Notch receptor undergoes proteolytic cleavage by a-disintegrin-and-metalloproteinase 10/17 (ADAM10/17) (S2 cleavage) and the γ -secretase complex (S3 cleavage), causing the release of the Notch intracellular domain (NICD). NICD then enters the nucleus and binds to the DNA-binding protein CSL (CBF-1 (RBPJ)/suppressor of hairless/Lag1), which recruits mastermind-like protein (MAML) to activate the transcription of Notch target genes including Hes and Hey families.^{1,2}

Three members of the Delta family (DLL1, DLL3, DLL4) are encoded on chromosome 6q27 (6: 170,282,200–170,291,078), 19q13.2 (19: 39,498,947–39,508,469) and 15q15.1 (15: 40,929,340–40,939,073), respectively. Prior to trafficking to the cell surface, DLLs undergo O-fucosylation mediated by Lunatic Fringe (Lfng) in the Golgi apparatus, which enhances^{4,5} or attenuates^{6,7} DLL-Notch signaling. Membrane DLLs bind to the EGF repeats of Notch receptors, subsequently triggering Notch signaling “in trans” through endocytic “pulling”. After endocytosis, internalized DLLs can undergo proteasomal/lysosome degradation or can be recycled back to the plasma membrane.^{2,8} The intracellular endocytosis/recycling events of DLLs are dependent on the ubiquitination of the intracellular domain of DLL (DICD).^{9,10} The Notch regulator Numb prevents DLL degradation, promoting their recycling back to the cell surface¹¹ (Figure 1B).

In addition to activating Notch signaling, DLLs negatively regulate Notch signaling through a variety of mechanisms (Figure 1C). Like Notch receptors,

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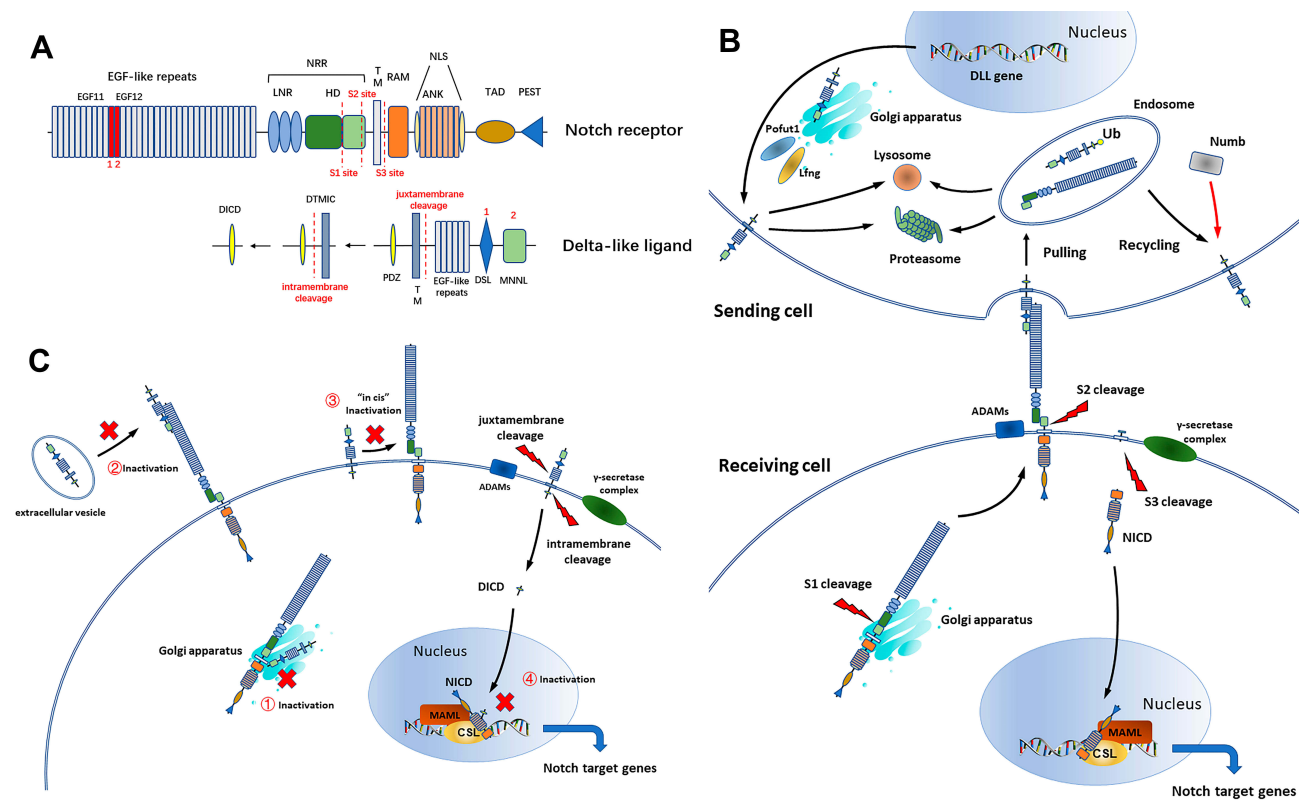


Figure 1 (A) Structure of DLL and the Notch receptor. The full-length Notch receptor consists of epidermal growth factor (EGF) repeats, a negative regulatory region (NRR), a transmembrane domain (TM), an RBP-association module (RAM) domain, seven ankyrin (ANK) repeats, two nuclear localization signals (NLS), a trans-activation domain (TAD) and a C-terminal proline, glutamate, serine, and threonine-rich (PEST) domain. The full-length DLL consists of an N-terminal domain of Notch ligand (MNNL), a Delta/Serrate/LAG-2 (DSL) domain, 6–8 EGF repeats, a TM and a post-synaptic density protein ligand (PDZ) domain (excluding DLL3). Adapted with permission from Xiu, M. X. & Liu, Y. M. The role of oncogenic Notch2 signaling in cancer: a novel therapeutic target. *Am J Cancer Res.* 2019;9(5):837–854.³ The core interactions between DLL and the Notch receptor are DSL-EGF11 and MNNL-EGF12. The full-length DLL can undergo juxtamembrane and intramembrane cleavage, releasing DICD. The proteolytic cleavage sites of DLL and the Notch receptor are marked. (B) DLLs activate Notch signaling. (C) DLLs inhibit Notch signaling. ① DLL3 inhibits Notch activation in the Golgi apparatus. ② Extracellular vesicle-containing DLL4 binds to Notch receptors and inhibits Notch signaling. ③ DLLs bind to Notch receptors at the cell surface and inhibit Notch signaling “in cis”. ④ DICD prevents the formation of NICD-CSL-MAML complex.

DLLs undergo proteolytic cleavage by ADAMs within their juxtamembrane (the cleavage site is 10 aa N-terminal to the transmembrane domain) and subsequently by the γ -secretase complex within their transmembrane regions (the cleavage site is unknown), releasing DTMIC (delta-like transmembrane and intracellular domain) and DICD.^{12–14} DICD is 100–150 aa in length and harbors a putative PDZ domain, predicted to couple DICD to membrane-bound proteins for DLL trafficking¹⁰ and to the JUN proto-oncogene and NICD-CSL-MAML complex for the nuclear regulation of target gene transcription.^{15–17}

Notch signaling in cancer can be oncogenic or tumor-suppressive.^{3–20} The roles of DLLs as an important family of Notch ligands, have not been systematically elucidated. In this review, we provide a comprehensive overview of the role of DLLs in cancer and propose novel safe and effective DLL-targeted treatment strategies.

Delta-Like Ligands in Cancer

DLLs are aberrantly expressed in a range of human cancers and influence cancer progression. To date, the roles of DLLs have been extensively studied in lung cancer, liver cancer, gliomas and breast cancer. These tumors have special properties such as neuroendocrine activity, viral infection or hormone secretion, which are closely related to DLLs. Furthermore, several DLL-targeted therapy strategies for the treatment of these cancer types have been proposed and are currently in pre- or clinical trials. We herein review recent progress in our understanding of DLLs in these four cancer types, and discuss the major roles of DLLs in other cancers.

Delta-Like Ligands in Lung Cancer

DLL1 plays a tumor-suppressive role in lung cancer. In the bone marrow of lung cancer patients, DLL1 is poorly expressed, whilst another Notch ligand Jagged1 is over-expressed in dendritic cells.²¹ Through the interactions

between dendritic cells and CD8⁺ T-cells, Jagged1-Notch signaling activates PD-1, impairing the activation of CD8⁺ T-cells. The overexpression of DLL1 by clustered DLL1 downregulates Jagged1-Notch signaling and decreases PD-1 expression, leading to a recovery in anti-tumor T-cell responses and increased T-cell infiltration.²¹ T-cell responses supported by DLL1 inhibit the growth and vascularization of lung tumors, revealing new avenues to modulate tumor immunity.^{21–23} In addition, DLL1 overexpression in small cell lung cancer (SCLC) cells improves the efficacy of chemotherapeutic and oncogene-targeted agents, leading to cell apoptosis and cell cycle arrest.²⁴

The majority of non-small cell lung cancer (NSCLC) tumors (69/84 in one study) exhibit DLL3 overexpression.²⁵ High DLL3 expression predicts poor overall survival (OS) in lung adenocarcinoma, playing an opposing prognostic role to DLL1.²⁶ In high-grade neuroendocrine lung tumors (including small cell lung cancer (SCLC) and large-cell neuroendocrine cancer (LCNEC)), DLL3 is overactive at the tumor cell surface and positively correlates with achaete-scute homolog-1 (ASCL1), a member of the BHLH family of transcription factors that is involved in neuroendocrine cell fate decisions.^{27–29} ASCL1 activates DLL3 by binding to special E-box sites in the DLL3 promoter, as observed in the dorsal neural tube.³⁰ In a recent study, LCNEC was divided into two molecular phenotypes based on the expression of DLL3 and ASCL1.³¹ Type I LCNEC with high ASCL1 and DLL3 expression exhibited a neuroendocrine profile with similarity to SCLC tumors, whilst type II LCNEC with low levels of ASCL1 and DLL3 exhibited reduced levels of neuroendocrine markers and markedly differed from SCLC tumors. The expression of DLL3 and ASCL1 may form a precise delineation of the molecular boundaries between different neuroendocrine tumors, aiding the identification of the respective molecular subtypes.^{29,31} In addition to ASCL1, thyroid transcription factor-1 (TTF1), a target of ASCL1, is overexpressed in SCLC tissue and positively correlates with DLL3 expression.^{32,33} The combination of DLL3 and TTF1 overexpression can predict poorer OS in SCLC patients.³² Further studies highlighted TTF1 as a surrogate DLL3 immunohistochemistry marker to identify DLL3-positive SCLC tissues. This may improve the precise identification of DLL3-responsive tumors for precision therapy approaches.³³

Recently, DLL3 has been detected in circulating tumor cells (CTCs) and in blood samples from SCLC

patients.^{34,35} SCLC patients with DLL3-positive CTCs show decreased OS and progression-free survival (PFS) compared to DLL3-negative patients. The percentage of DLL3-positive CTCs is a liable dynamic real-time biomarker that predicts cancer progression. It significantly declines after chemotherapy but increases in cases of tumor relapse.³⁵

Due to the strong expression of DLL3 on the surface of SCLC cells, the DLL3-targeted investigational product Rova-T was designed to bind to DLL3-positive SCLC cells, exerting further cytotoxic effects (see details in 4.4.).³⁶ Other DLL3-targeted products include AMG 119 (HLE BiTE[®] antibody) and AMG 757 (chimeric antigen receptor (CAR)), designed to activate and redirect CD3-positive T cells to DLL3-expressing tumors to induce cell apoptosis.³⁶ Two Phase I clinical trials NCT03392064 and NCT03319940 have evaluated the safety, tolerability and efficacy of AMG 119 and AMG 757 for SCLC. In addition to targeted drugs, near infrared photoimmunotherapy (NIR-PIT) is a novel DLL3-targeted strategy for SCLC treatment that employs an antibody-photosensitizer conjugate followed by NIR light exposure to damage DLL3-positive tumor cells. In vivo experiments have confirmed that DLL3-overexpressing SCLC tumors are immediately destroyed upon NIR-light exposure.³⁷

Immunohistochemistry is used to assess DLL3 expression from SCLC biopsies and predict the effects of DLL3-targeted agents; however, several practical limitations remain.³⁸ The application of immuno-positron emission tomography (immunoPET) can non-invasively provide real-time information regarding the status of in vivo DLL3 expression in tumors, facilitating the selection of patients for treatment with DLL3-targeted agents. An 89Zr-labeled, DLL3-targeted immunoconjugate leveraging the humanized antibody, SC16, has been developed to serve as a companion diagnostic immunoPET agent, with a promising performance in preclinical mouse models of SCLC.³⁸

The role of DLL4 expression in lung cancer is controversial. DLL4 expression is accompanied by elevated hypoxia inducible factor-1 α (HIF1 α), microvessel density (MVD) and vascular endothelial growth factor (VEGF), promoting tumor angiogenesis.^{39–41} Conversely, endothelial DLL4 overexpression in Lewis lung cancer (LLC)-bearing mice decreases the expression of VEGF-R2, which reduces the vascular sensitivity to VEGF-A, leading to decreased endothelial density. The tumor vasculature is more mature and stable upon DLL4 stimulation, enhancing

the delivery of chemotherapy drugs to the tumor site.⁴² In addition, DLL4-expressing endothelial cells (ECs) can inhibit the proliferation of neighboring NSCLC cells through the activation of Notch1/PTEN signaling in NSCLC cells, suggesting endothelial DLL4 has a tumor-suppressive role in lung cancer.⁴³

Delta-Like Ligands in Liver Cancer

Activated DLL1-Notch signaling in HCC is maintained through the high expression of Pofut1, a glycosyltransferase that enhances the interaction between DLL1 and Notch receptors. Pofut1 silencing is a promising strategy for inhibiting DLL1/Notch signaling, which substantially represses the migration and invasion of HCC cells.⁴⁴

Hepatitis viruses play an important role in the regulation of DLL expression. DLL3 is expressed in the cytoplasm of normal hepatocytes, but is silenced in HCC by DNA methylation and histone acetylation induced by the hepatitis B virus (HBV).⁴⁵ Histone deacetylase (HDAC) inhibitors such as TSA effectively restore DLL3 expression in HCC cells. The reactivation of DLL3 inhibits HCC cell growth and induces cell apoptosis independently of Notch1, suggesting that DLL3 has a tumor-suppressive role through Notch-independent mechanisms.⁴⁵⁻⁴⁷ In contrast, DLL3 gene expression is 7.22-fold higher in circulating CD133+ cells from hepatitis C virus (HCV)-infected HCC patients compared to cells from control patients, however the effects of DLL3 overexpression on HCV-associated HCC tumors remain undefined.⁴⁸

Unlike DLL3, DLL4 is upregulated in HCC by HBV infection and activates oncogenic Notch1-Hes1 signaling. DLL4 silencing has been shown to reduce HCC cell viability, leading to G1-phase cell cycle arrest, with no effects on viral replication.⁴⁹ Conversely, Kunanopparat et al showed that DLL4 is able to suppress HBV replication by an unknown mechanism, independent of anti-viral cytokine production or enhanced T cell immunity.⁵⁰ Further investigations should focus on the association between DLLs and hepatitis viruses, to improve our understanding of hepatitis virus-associated HCC and the development of novel targeted therapies.

Delta-Like Ligands in Gliomas

Overexpressed DLL1 inhibits neural differentiation and contributes to the development of medulloblastoma tumors.⁵¹ In glioma cells, DLL1 forms a positive feedback loop with Notch1 to promote cell survival and growth.⁵² DLL1 prevents glioma cells from transdifferentiating into

vascular ECs, furtherly maintaining their population.⁵³ DLL1 or Notch1 silencing inhibits the proliferation and survival of glioma cells, and significantly prolongs the survival of glioma-bearing mice.^{52,54}

DLL3 expression in *IDH* mutant gliomas, particularly in 1p/19q co-deleted subsets is higher than that of *IDH* wild-type glioblastoma.⁵⁵ The treatment of *IDH* mutant glioma cell lines with Rova-T can effectively induce the cytotoxicity of DLL3-expressing cells.⁵⁵ In *IDH* wild-type glioblastoma, DLL3 is mainly expressed in the tumor lesions contacting the subventricular zone (SVZ) but not involving the cortex, which is a prognostic marker of poor PFS.⁵⁶ However, in high-grade gliomas, DLL3 is defined as a proneural signature gene that is associated with longer survival, with DLL3/Notch signaling a major determinant of tumor growth.⁵⁷ In vitro experiments indicate that DLL3 expression is downregulated in glioma cells, the recovery of which can inhibit the survival, proliferation and invasiveness of glioma cells.^{58,59} The pro-tumoral and tumor suppressor effects of DLL3 may be dependent on its distribution in tumor cells (see in 3.2.).

DLL4 localizes to the cytoplasm and membranes of gliomas ECs but rarely distributes in glioma cells or normal brain tissue.⁶⁰⁻⁶³ Increased DLL4/Notch expression in the large and mature vessels of gliomas promotes a quiescent vascular phenotype that reduces the density of the tumor vasculature.^{61,64-66} However, DLL4 in some cases is associated with high MVD and is expressed in some microvascular formations of gliomas, including delicate capillary-like,^{67,68} garland-like,⁶⁸ sprouted and clustered^{60,69} and glomeruloid cells.^{62,68,69} These findings indicate a complex relationship between DLL4 and tumor angiogenesis. Its potential involvement mechanisms are discussed in 3.3.2.

Delta-Like Ligands in Breast Cancer

DLL1 is overexpressed in breast cancer (BC) and is associated with poorer prognosis, particularly in the ER α + luminal subtype.⁷⁰ Estrogen stabilizes DLL1 expression by inhibiting the proteasomal and lysosomal degradation of DLL1, which promotes the growth and angiogenesis of ER α + luminal tumors. Silencing ER α expression in ER α + BC cells significantly decreases DLL1 expression and prevents Notch activation, suggesting that blocking the estrogen/DLL1/Notch axis is a potential targeting strategy for ER α + BC.⁷⁰ Recently, in vitro experiments demonstrated the anti-tumor effects of DLL1 knockdown on human BC cell lines were mediated through inhibiting

the proliferation and survival of luminal A cells, the clonogenic growth of luminal B cells, and the migration and invasion of triple-negative, claudin-low cell lines.⁷¹ miRNA-130b was identified as a potential inhibitor of DLL1 in BC through binding to its 3'UTR region (217–224 bp) suppressing its translation. The inhibition of DLL1 by miR-130b mimics effectively reduces the migration and invasion of BC cells.⁷²

DLL4 is overexpressed in the plasma and tumor tissue of BC patients and is associated with a poor outcome, metastasis and drug resistance.^{73–77} The inhibition of DLL4 by RGD peptide-modified lipid nanoparticles (RGD-LNPs) encapsulating siRNA prolongs the OS of mouse models of BC with lung metastasis.⁷⁸ In addition, lung metastasis in BC can be inhibited by the anti-cancer therapeutic peptides AD-01 and ALM201, which down-regulate DLL4 and Notch4.⁷⁴

Preventing DLL4 activation in BC using antibody-based drugs represents another potential DLL4-targeting strategy, exhibiting potent anti-tumor activity in pre-clinical studies.^{79,80} In addition, a combination of anti-DLL4 and anti-VEGF treatment in BC using bispecific antibodies not only induces tumor cell apoptosis, but also interferes with tumor angiogenesis, thus inhibiting BC progression in vivo.^{81,82}

Delta-Like Ligands in Other Cancers

In addition to the above four cancers, the role of DLLs in other cancer types is shown in Table 1. Overexpressed DLL4 is the main ligand that activates oncogenic Notch signaling and is widely reported to predict a poor clinical outcome in pancreatic cancer (PC), gastric cancer (GC) and clear cell renal cell cancer (ccRCC).^{83–92} Pre-clinical studies show that blockade of endothelial DLL4 (mDLL4) by anti-mouse DLL4 antibodies (anti-mDLL4) HMD4-2 or 21R30 inhibits neovascularization and the growth of PC in vivo, suggesting DLL4-Notch signaling is a potential target for PC treatment.^{93,94} In GC, DLL4 is mainly expressed in the membranes of tumor cells as opposed to the tumor stroma.⁸⁹ DLL4/Notch signaling maintains the self-renewal and invasion ability of GC cells, which can be inhibited by DLL4 knockdown.^{88,95,96} In contrast, DLL4 is expressed in the vascular endothelium of ccRCC. Endothelial DLL4 in ccRCC can on one hand promote tumor angiogenesis through VEGF activation,^{92,97–99} or activate Notch signaling in tumor cells, thus inducing hematogenous metastasis.⁹⁷ In Ewing's sarcoma, DLL4/Notch signaling is activated in the perivascular stroma of

tumors derived from bone marrow (BM), inducing the differentiation of BM cells into pericytes/vascular smooth muscle cells (vSMCs) that provide structural support to the vessels, permitting tumor vasculature maturation and functionality.^{100–102} Blocking tumor DLL4 (hLL4) with YW152F, an anti-human DLL4 antibody, blocks pericyte/vSMC formation, negatively regulating vessel functionality and vascular expansion.¹⁰¹

Different Ligands Act Through Alternate Mechanisms

The roles of the three Notch DLLs in cancer are variable. Through complex mechanisms, DLLs can activate or inactivate Notch signaling under different conditions, which influences cancer progression. Prior to the identification of appropriate DLL-targeted therapies, it is necessary to understand the specific functions of different DLL ligands in cancer.

DIII

As a membrane ligand, DLL1 binds to the Notch receptor and activates Notch signaling in cancer cells “in trans”, enhancing tumorigenesis.^{54,70,103} Prior to its translocation to the plasma membrane of cancer cells, cytoplasmic DLL1 undergoes Pofut1-dependent O-fucosylation modulation,⁴⁴ and is transported through vesicular trafficking, supported by the Actin-related protein2/3 complex (ArpC), a regulator of the actin cytoskeleton.¹⁰⁴ ArpC knockdown decreases DLL1 expression at the cell surface, impairing the stem-cell phenotype of cancer cells and abolishing their tumorigenicity.¹⁰⁴

Notch1 signaling is silenced by the hypermethylation of the DLL1 promoter in GC samples (particularly in *H. pylori*-positive tissue), which constitute specialized characteristics of the diffuse histotype.^{105–107} The role of DLL1 methylation-induced Notch1 inactivation in GC is uncertain, and the overexpression of N1ICD maintains a cancer stem cell-like phenotype of GC cells.¹⁰⁷

Although DLL1 plays an oncogenic role in cancer, its potential tumor-suppressive effects have been identified. With DLL1 interventions, tumor neovascularization is inhibited by the loss of tumor ECs derived from cancer stem cells (CSCs),⁵³ the inhibition of VEGF-R2-mediated VEGF signaling^{108,109} and increased hypoxia and tumor cell necrosis.^{108,109} In the hematopoietic environment of tumors, the downregulation of DLL1 impairs T cell development, leading to a loss of anti-tumor T cell responses.²²

Table I The Role of DLLs in Other Cancers

| Cancer Types | Notch Ligand | Functions | Observation | References |
|-----------------------|----------------------|---|--|---------------------------------|
| Pancreatic carcinoma | DLL1 DLL3 DLL4 | Tumor-suppressive Oncogenic Oncogenic | High DLL1 expression is associated with higher survival probabilities. Activated DLL3 drives Notch signaling to promote PC cell growth. High DLL4 expression is associated with poor OS and DFS and severe clinicopathological characteristics. Activated DLL4 promotes the EMT process and induces chemoresistance of PC cells. | 174 175 83–87 94,120 |
| Gastric carcinoma | DLL1 DLL4 | Uncertain Oncogenic | Activated DLL1 promotes tumor growth in vivo and inhibits GC cell proliferation in vitro. High DLL4 expression is associated with poor OS. DLL4 is critical for the self-renewal and tumorigenicity of GC cells by activating Notch1 signaling. Activated DLL4 promotes the growth, proliferation and invasion of GC cells. | 107 88,89 88 95,121 |
| Melanoma | DLL1 DLL3 | Oncogenic Tumor-suppressive Oncogenic | Activated DLL1 promotes the adhesion and metastasis of melanoma cells by upregulating N-cadherin. Activated DLL1 inhibits tumor growth in vivo by downregulating VEGFR2-mediated neovascularization. LPS-induced inflammation activates DLL3, which promotes the migration, invasion and EMT process of melanoma cells. DLL3/Notch2/Notch4 pathway is critical for the survival and growth of melanoma cells. DLL3/MAPK pathway is critical for the proliferation and migration of melanoma cells. | 103 108 176 177 178 |
| Osteosarcoma | DLL1 | Tumor-suppressive | Activated DLL1 downregulates the multi-chemoresistance of OS cells. | 153 |
| Endometrial carcinoma | DLL3 | Oncogenic | High DLL3 expression is associated with poor OS. | 179 |
| Bladder carcinoma | DLL3 DLL4 | Oncogenic Oncogenic | High DLL3 expression is associated with poor OS and PFS. High DLL4 expression positively correlates with MVD, VEGF and tumor vessel maturation. | 113 139 |
| Ovarian carcinoma | DLL3 DLL4 | Oncogenic Oncogenic | Notch2/Notch3/DLL3/MAML1/ADAM17 axis is activated in OC and is associated with poor OS. High DLL4 expression is associated with poor OS and severe clinicopathological characteristics. DLL4 is critical for the growth, proliferation and migration of OC cells and tumor angiogenesis. | 180 140,181 140 |
| T-ALL | DLL4 | Oncogenic | DLL4/Notch3 pathway is critical for the survival, proliferation and tumorigenicity of T-ALL cells. DLL4 is critical for the growth of T-ALL cells. | 131 182 |
| Ewing's sarcoma | DLL4 | Oncogenic | Active DLL4 induces vasculogenesis by promoting the formation of bone marrow-derived pericytes. | 100–102 |
| Medulloblastoma | DLL4 | Oncogenic | DLL4 is critical for cancer stem cell maintenance under hypoxia. | 145 |
| Cervical carcinoma | DLL4 | Oncogenic | High DLL4 expression is associated with poor OS, DFS and pelvic lymph node metastasis. | 183 |
| Esophageal carcinoma | DLL4 | Oncogenic | DLL4 is critical for the growth, metastasis, anti-apoptosis, invasion and migration of cancer cells by activating PI3K/Akt/E-cadherin pathway. | 125 |

(Continued)

Table I (Continued).

| Cancer Types | Notch Ligand | Functions | Observation | References |
|--------------------------------|--------------|--------------------------------|--|----------------------------------|
| Colon carcinoma | DLL4 | Oncogenic Tumor-suppressive | DLL4 is critical for the growth, survival and angiogenesis of tumor. DLL4 is activated in response to chemotherapy and reduces tumor angiogenesis. | 135,156 184 |
| Cholangiocarcinoma | DLL4 | Oncogenic | High DLL4 expression is associated with poor OS. | 185 |
| Gallbladder carcinoma | DLL4 | Oncogenic | High DLL4 expression is associated with poor OS and severe clinicopathological characteristics. | 185,186 |
| Squamous cell head-neck cancer | DLL4 | Tumor-suppressive | High DLL4 expression is associated with improved LRFS and reduced radio-resistance. | 187 |
| Nasopharyngeal carcinoma | DLL4 | Oncogenic | High DLL4 expression positively correlates with VEGF and is associated with poor DFS. | 188 |
| Renal cell carcinoma | DLL4 | Oncogenic | High DLL4 expression is associated with poor OS and severe clinicopathological characteristics. High DLL4 expression positively correlates with MVD and the expression of VEGF. DLL4 is critical for the proliferation, migration and formation of tumor ECs and tumor angiogenesis. Endothelial DLL4 promotes the migration, invasion and metastasis of RCC cells by activating the Notch/Hey1/MMP9 pathway. | 90–92 97,98 92,98,99 97 |

Abbreviations: OS, overall survival; DFS, disease-free survival; PFS, progression-free survival; LPS, lipopolysaccharide; EMT, epithelial-mesenchymal transition; MAPK, mitogen-activated protein kinase; T-ALL, T-cell acute lymphoblastic leukemia; LRFS, locoregional relapse-free survival; ECs, endothelial cells.

Clustered DLL1 increases tumor infiltration by immune cells, but inhibits tumor neovascularization. Importantly, multivalent DLL1 therapy is a safe therapeutic intervention that does not stimulate tumor growth or organ abnormalities, the benefits of which now warrant further investigation.^{22,23}

DII3

Unlike other ligands, DLL3 is considered as an inhibitor of Notch signaling. On one hand, in a cell autonomous manner, DLL3 localizes to the Golgi apparatus and binds to DLL1 and/or the full-length Notch1 receptor, preventing their modification and promoting their degradation.^{6,110} On the other hand, membrane DLL3 binds to Notch receptors in the same cell and prevents their activation “in cis”.¹⁶ Notably, the inhibitory effects of DLL3 are dependent on the presence of Lfng, which mediates the O-fucosylation of DLL3 EGF-like repeats 2 and 5.^{6,7}

In tumors, a range of DLL3 functions have been described (Figure 2). DLL3 is not expressed on the surface of normal cells, but is highly expressed on the surface of tumor cells, particularly those with histopathologic features of neuro- or neuro-endocrine origin, including *IDH* wild-

type and mutant gliomas,^{55,56} neuroendocrine lung cancer,²⁸ neuroendocrine prostate cancer (NEPC),¹¹¹ gastrointestinal neuroendocrine cancer (GI-NEC)¹¹² and small cell bladder cancer (SCBC).¹¹³ DLL3 is upregulated at the transcriptional level in neuroendocrine tumors by the *ASCL1* oncogenic driver, and is predominantly expressed in metastatic and aggressive disease phenotypes.^{31,111,113} mRNA microarray analysis revealed that membrane DLL3 can activate Notch signaling “in trans” like other ligands in *IDH* wild-type gliomas.⁵⁶ In GI-NEC, SCLC and SCBC, DLL3 shows a cytoplasmic distribution in tumor cells (reported as Golgi

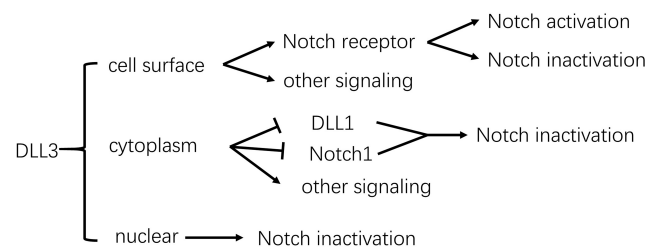


Figure 2 Distribution and function of DLL3 in tumor cells. Notch signaling is inhibited by DLL3 at the cell surface, Golgi apparatus, and in the nucleus of tumor cells, as well as being activated by membrane DLL3 “in trans”. In addition, DLL3 regulates other signaling cascades independently of Notch, exerting as-yet undefined functions.

apparatus-localized in a single SCLC study¹¹⁴) but how this influences Notch signaling is unknown.^{27,28,112,113,115–117} In LLC cells, DLL3 localizes to the cell nuclei, inhibiting Notch signaling at the transcriptional level. Akt signaling is activated by DLL3, which promotes LLC cell survival and reduces cell apoptosis.²⁵

Dll4

DLL4 in Regulation of Cell Behaviors

DLL4 overexpression in cancer leads to anti-angiogenic effects that restrain excessive vascular sprouting, through triggering a negative feedback with VEGF.^{118,119} The effects of DLL4 are profound, not only affecting tumor angiogenesis, but also regulating cell behavior. DLL4 permits sustained Notch activation and promotes the crosstalk between tumor cells (T-T),^{49,73,88,120–125} ECs and ECs (E-E),^{98,126–128} tumor cells and ECs (T-E)^{43,97,129–132} (Figure 3).

The three main oncogenic pathways including NF-κB, PI3K/Akt and MMP-2/9 signaling are potential therapy targets that are activated by DLL4/Notch signaling through T-T and T-E interactions. In SCLC, blocking DLL4 expression prevents the interaction of NICD with the p65, p50, and RelB subunits of NF-κB, thus inhibiting the liver micro-metastasis of SCLC cells.¹²⁴ In T-cell acute lymphoblastic leukemia (T-ALL) and colorectal cancer cells, Notch3 signaling is triggered by endothelial DLL4 and increases NF-κB DNA-binding, promoting tumor growth.¹³¹

DLL4/Notch1 signaling enhances Akt phosphorylation and the formation of the Akt/IKK-α complex in glioma cells, thus activating NF-κB and β-catenin signaling. β-catenin silencing and/or Akt inhibitor treatment abrogates glioma cell migration and invasion.¹²² In addition, the knockdown of DLL4 in esophageal cancer cells attenuates Akt phosphorylation and downregulates E-cadherin expression, abolishing cell growth and metastasis.¹²⁵

Through T-T interactions, the expression and secretion of MMP2 and MMP9 are upregulated in response to DLL4/Notch signaling, which contributes to cancer cell migration and invasion.^{121,123} Through T-E interactions, endothelial DLL4 activates Notch1-MMP2/9 signaling in cancer cells, promoting cell proliferation, migration and angiogenesis.¹³⁰

DLL4 in Tumor Vasculature

Endothelial DLL4 is expressed in VEGF-sensitive tip cells and activates Notch signaling in adjacent VEGF-insensitive stalk cells, which in turn restricts VEGF-dependent neoangiogenesis, suppresses the tip cell fate and facilitates the conversion of tip cells to stalk cells.^{119,133} DLL4-overexpressing tumors exhibit unique vascular characteristics, including fewer but larger vessels, improved vessel perfusion and decreased tumor hypoxia and necrosis.^{42,68,134,135} MVD, as an indicator of the degree of tumor angiogenesis and the proliferation state of tumor ECs, negatively correlates with the expression of DLL4 in tumors.^{60,61,67,68,120,131,135–137} However, in

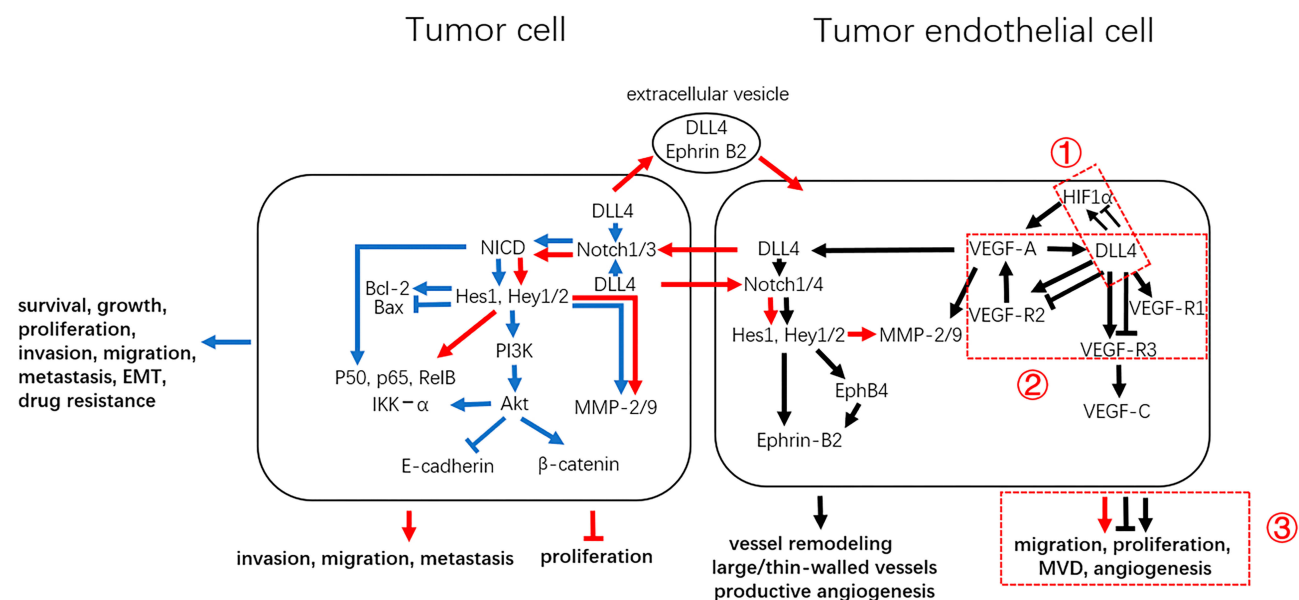


Figure 3 DLL4 regulates the behavior of tumor cells and tumor ECs. DLL4-mediated signal transduction between tumor cells, tumor cells and ECs, and EC cells are shown in blue, red, and black, respectively. The three contradictions regarding DLL4 activity are represented by dashed boxes: (1) DLL4 and HIF1α; (2) DLL4 and VEGF signaling; (3) DLL4 and EC cell behavior.

several cases, MVD is positively associated with DLL4 expression, which contradicts the effects of DLL4 on inhibiting neovascularization.^{39–41,50,63,75,83,92,97,98,138,139} Herein, we discuss the possible mechanisms responsible for DLL4-induced functional and productive angiogenesis, and for the first time describe the potential effects of DLL4 on promoting tumor neovascularization.

VEGF Signaling

Through methylation of the VEGF-R2 promoter, DLL4 directly inhibits VEGF-R2 expression in downstream stalk cells, which blocks VEGF-A/VEGF-R2 signaling and suppresses tumor angiogenesis.¹⁴⁰ Surprisingly, in some tumors, a positive correlation between the expression of DLL4 and VEGF-R2 exists, indicating that DLL4 can also activate the VEGF-A/VEGF-R2 axis.^{39,50,87,91,130} VEGF-R1, which lacks potent signaling activity in ECs, can be upregulated by DLL4.^{39,42,141} In addition, VEGF-R3 can be activated⁴² or inhibited^{141,142} by DLL4 in tumors, which has potential effects on VEGF-C/VEGF-R3-mediated angiogenesis.

Ephrin-B2/EphB4 Signaling

In addition to VEGF signaling, DLL4 affects tumor endothelial function by activating Ephrin-B2/EphB4 signaling.^{126,127} Ephrin-B2 is directly downstream of VEGF and DLL4, and the VEGF/DLL4/Notch4 axis activates Ephrin-B2 reverse signaling to induce abnormal vessel remodeling in liver tumors through elevated Ephrin-B2.¹²⁷ In U87-DLL4 tumors, Ephrin-B2 reverse signaling is activated by elevated EphB4 in response to anti-VEGF therapy.¹⁴¹ The inhibition of Ephrin-B2/EphB4 signaling through anti-Ephrin-B2 antibodies, soluble Ephrin-B2 or soluble EphB4 leads to increased EC proliferation, reduced vessel size and a loss of tumor growth, which mimics the non-productive angiogenesis induced by DLL4 blockade.^{128,141} However, Djokovic et al found that soluble EphB4 therapy in RIP1-Tag2 tumors leads to reduced tumor vessel density, suggesting the existence of a positive feedback loop between Ephrin-B2/EphB4 and VEGF signaling.¹⁴²

Intratumor Hypoxia

Intratumor hypoxia is a major driver of tumor angiogenesis,¹⁴³ which can be inhibited by DLL4 stimulation.^{68,100,101,134,135,141,144} However, a positive correlation between DLL4/Notch signaling and intratumor hypoxia also has been found in clinical cases and in vitro,

which may promote tumor angiogenesis.^{39,42,63,145} Notably, because hypoxia is a critical inducer of VEGF, DLL4 indirectly determines changes in VEGF expression through its effects on HIF1 α .^{42,142}

The Transportation of Extracellular Vesicles Containing DLL4

The crosstalk of tumor cells and tumor ECs mediated by DLL4 is not only dependent on cell-cell interactions, but also on vesicles. In HCC, DLL4 and Ephrin-B2 are secreted by HCC cells and are transported by microvesicles and exosomes to ECs, enhancing migration and angiogenesis.¹²⁹ In U87 tumors, DLL4-containing exosomes are incorporated into the plasma membrane of ECs and Notch receptors are downregulated. The inhibition of Notch signaling in ECs leads to nonproductive angiogenesis and increased MVD, suggesting that tumor cell-derived vesicles containing DLL4 have a positive effect on tumor neovascularization.¹⁴⁶

Targeted DLL Therapy: Is It Effective and Safe?

According to the characteristics of each DLL ligand, an array of DLL-targeting strategies have been proposed. Recombinant DLL proteins can be used to exert tumor-suppression, whilst a large number of pre- or clinical studies using DLL-targeting drugs have been reported (Table 2). However, some non-negligible problems regarding drug safety have been encountered, highlighting how both the safety and effectiveness of DLL-targeted strategies must be considered during tumor therapy.

Recombinant Proteins

The majority of soluble recombinant DLL proteins are formed from specific Notch-receptor binding domains (DSL/DSL~EGF2-3/extracellular domain) fused to IgG Fc- or His-tags.^{21,66,68,109,147–149} Due to the lack of multivalent interactions between Notch receptors and their ligands, and their inability to induce Notch ligand endocytosis, these monovalent soluble forms of DLL1/4 can be classed as Notch inhibitors.^{21,66,124,149} However, soluble DLL1/4 can also activate Notch signaling, indicating their uncertain effects on the Notch signaling axis.^{68,118,148}

To exert a positive role in anti-tumor T cell immunity, a multivalent clustered DLL1 was designed consisting of

Table 2 Multiple miRNAs Affect Cancer Progression by Regulating DLLs

| MiRNAs That Have Tumor-Suppressive Functions | | | | | |
|--|--------------|-----------------------------|--|------------------------------|------------|
| miRNA | Notch Ligand | Tumor/Cell Type | Observation | Binding Region at DLL 3'-UTR | References |
| miRNA-34a | DLL1 | Medulloblastoma | MiRNA-34a inhibits the proliferation and growth of MB cells and induces cell differentiation and apoptosis by downregulating DLL1. | Not shown | 51 |
| | | Choriocarcinoma | MiRNA-34a inhibits the proliferation and invasion of CC cells by downregulating DLL1. | CACUGCC | 151 |
| miRNA-34 | DLL1 | Neuroblastoma | MiRNA-34 family members induce NB cell differentiation by downregulating DLL1, which leads to cell proliferation arrest. | Not shown | 152 |
| miRNA-130b | DLL1 | Breast carcinoma | MiRNA-130b inhibits the invasion and migration of BC cells by downregulating DLL1. | UUGCACU | 72 |
| miRNA-182 | DLL4 | Renal cell carcinoma | MiRNA-182 inhibits the proliferation and migration of RCC cells by downregulating DLL4. | Not shown | 189 |
| miRNA-518d | DLL3 | Small cell lung carcinoma | MiRNA-518d inhibits the proliferation, migration and EMT process of SCLC cells by downregulating DLL3. | C-UC-CUCUAGA | 154 |
| miRNA-30a | DLL4 | Renal cell carcinoma | MiRNA-30a inhibits the proliferation and migration of tumor ECs by downregulating DLL4. | UGUUUAC | 98 |
| MiRNAs That Have Oncogenic Functions | | | | | |
| miRNA-18 | DLL3 | Gliomas | MiRNA-18 promotes the proliferation and self-renewal of glioma cells by downregulating DLL3. | AGGGGAGGCAGAGGGGCAG | 58 |
| miRNA-34a | DLL1 | Gliomas | MiRNA-34a induces GSC transdifferentiation by downregulating DLL1. | Not shown | 53 |
| | | Osteosarcoma | MiRNA-34a promotes multi-chemoresistance of OS cells by downregulating DLL1. | CACUGCC | 153 |
| | | Endometrioid adenocarcinoma | Overexpressed miRNA-34a downregulates Notch1 and DLL1 in EAC tissues. | Not shown | 190 |
| MiRNAs That Have Uncertain Functions | | | | | |
| miRNA-27b | DLL4 | Lung adenocarcinoma | MiRNA-27b inhibits DLL4 expression in tumor vasculature but promotes DLL4 expression in tumor cells. | Not shown | 191 |

Abbreviations: ECs, endothelial cells; GSC, glioma stem cell.

the extracellular domain of DLL1, IgG Fc-tags, biotinylated anti-Fc antibodies, and NeutrAvidin, which triggers Notch signaling in T cells.^{22,23} A soluble DLL1 protein containing a DSL domain and an arginine-glycineaspartate (RGD) motif (CRGDCGVRY) can also be targeted to Notch receptors on tumor EC cells, triggering DLL1-Notch signaling in the tumor vasculature, inhibiting angiogenesis.¹⁰⁹

MiRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that directly bind to the 3'-UTR of target mRNAs, leading to gene silencing.¹⁵⁰ Several miRNAs have been shown to be involved in the regulation of DLL with a range of multifaceted functions reported (Table 3).

MiRNA-34a is an important antagonist of DLL1 in tumors, which can be directly activated by p53.^{53,151} The

Table 3 The DLL-Targeted Drugs

| Molecular Inhibitor | Character | Target | Pre-Clinical Condition | Mechanism/Effect | References | Clinical Trials |
|------------------------|---------------------------------|--------|--|---|------------------------------------|--|
| SCI6LD6.5/ Rova-T | Antibody-drug conjugate (ADC) | DLL3 | High-grade neuroendocrine lung cancer IDH-mutant gliomas Neuroendocrine prostate cancer Small cell bladder cancer | Be internalized and trafficked to late endosomes in DLL3-expressing tumor cells and induce cytotoxicity. Target DLL3 antigen on glioma cell surface and induce cell apoptosis. Inhibit tumor growth by inhibiting the viability of DLL3-expressing tumor cells. Inhibit tumor growth and recurrence by targeting DLL3-expressing CSCs. | 28 55 111 113 | NCT01901653 NCT02674568 NCT02709889 NCT02819999 NCT03000257 NCT03026166 NCT03033511 NCT03061812 NCT03086239 NCT03334487 |
| AMG 119 | Chimeric antigen receptor (CAR) | DLL3 | Small cell lung cancer | Modify autologous T cells to express a DLL3-targeted chimeric antigen receptor (CAR) and ablate DLL3-positive tumor cells robustly | 36 | NCT03392064 |
| AMG 757 | Anti-DLL3 x CD3 BiTE® antibody | DLL3 | Small cell lung cancer | Redirect CD3-positive T cells to induce serial lysis of DLL3-positive tumor cells | 36 | NCT03319940 |
| Demcizumab (OMP-21M18) | Anti-hDLL4 antibody | DLL4 | Pancreatic cancer; colon cancer; breast cancer Colon cancer | Inhibit tumor growth and tumorsphere formation; reduce CSC frequency; delay tumor recurrence; restore drug sensitivity Inhibit tumor growth; reduce CSC frequency; induce tumor cell apoptosis | 94,155 156 | NCT01189929 NCT01189942 NCT01189968 NCT01952249 NCT02259582 NCT02289898 NCT02722954 |
| MEDI0639 | Anti-hDLL4 antibody | DLL4 | HUVECs | Induce immature neoangiogenic vessels in vivo | 157 | NCT01577745 |
| Enoticumab (REGN421) | Anti-hDLL4 antibody | DLL4 | Ovarian cancer Renal cell cancer | Inhibit tumor growth; induce nonfunctional tumor vasculature; reduce vascular perfusion No significant anti-tumor efficacy | 132,136 167 | NCT00871559 |
| MMGZ01 | Anti-hDLL4 antibody | DLL4 | Breast cancer | Inhibit tumor growth; reduce CSC frequency; induce nonfunctional tumor vasculature; inhibit the proliferation and EMT of BC cells; induce BC cell apoptosis | 79,80 | None |

(Continued)

Table 3 (Continued).

| Molecular Inhibitor | Character | Target | Pre-Clinical Condition | Mechanism/Effect | References | Clinical Trials |
|----------------------------|---|---------------|--|---|--------------------|---|
| YW152F | Anti-hDLL4 antibody | DLL4 | Breast cancer; colon cancer; lung cancer; | Inhibit tumor growth; induce nonfunctional tumor vasculature | 137 | None |
| Navicixizumab (OMP-305B83) | Anti-hDLL4 and anti-hVEGF bispecific antibody | DLL4 | None | None | None | NCT02298387 NCT03030287 NCT03035253 |
| HD-105 | Anti-hDLL4 and anti-hVEGF bispecific antibody | DLL4 and VEGF | Lung cancer; gastric cancer | Inhibit tumor progression and angiogenesis; induce tumor cell apoptosis | 169 | None |
| HB-32 | Anti-hDLL4 and anti-hVEGF bispecific antibody | DLL4 and VEGF | Breast cancer | Inhibit tumor growth and angiogenesis; inhibit BC cell proliferation; induce BC cell apoptosis | 81 | None |
| ABT-165 | Anti-hDLL4 and anti-hVEGF bispecific antibody | DLL4 and VEGF | Breast cancer; colon cancer; gliomas; pancreatic cancer | Inhibit tumor growth; induce nonfunctional tumor vasculature; reduce vascular perfusion; improve chemotherapy efficacy | 82 | NCT01946074 NCT03368859 |
| 21R30 | Anti-mDLL4 antibody | DLL4 | Pancreatic cancer | Inhibit tumor growth; delay tumor recurrence; restore drug sensitivity | 94 | None |
| HMD4-2 | Anti-mDLL4 antibody | DLL4 | Pancreatic cancer | Inhibit tumor growth and angiogenesis | 93 | None |
| REGN1035 | Anti-mDLL4 antibody | DLL4 | Ovarian cancer Renal Cell Cancer | Inhibit tumor growth; induce nonfunctional tumor vasculature Inhibit tumor growth; inhibit tumor angiogenesis combined with anti-VEGF treatment | 132,136 167 | None None |
| — | Anti-DLL4 antibody | DLL4 | Colon cancer Colon cancer; pharyngeal squamous cancer | Inhibit tumor growth; induce nonfunctional tumor vasculature; reduce vascular perfusion Inhibit tumor growth; induce nonfunctional tumor vasculature; reduce vascular perfusion; inhibit tumor cell proliferation; induce tumor cell apoptosis; increase radiosensitization; increase tumor necrosis | 192 135 | None |
| 3Nb3 | Anti-DLL4 nanobody | DLL4 | Gastric cancer HUVECs | Induce nonfunctional tumor vasculature; inhibit GC cell proliferation; induce GC cell apoptosis Induce nonproductive angiogenesis | 165 164 | None |

(Continued)

Table 3 (Continued).

| Molecular Inhibitor | Character | Target | Pre-Clinical Condition | Mechanism/Effect | References | Clinical Trials |
|----------------------|--------------------------------|--------------|----------------------------|--|------------|---------------------------|
| — | Anti-hDLL4 F(ab') ₂ | DLL4 | Colon cancer | Inhibit tumor growth | 162 | None |
| MvM03 and MGD03 | ADC | DLL4 | Breast cancer | Inhibit tumor growth and angiogenesis; inhibit BC cell proliferation; induce BC cell apoptosis | 173 | None |
| ALM201 (FKBPL/AD-01) | Therapeutic peptide | DLL4, Notch4 | Breast cancer | Inhibit the migration, invasion and lung metastasis of BC cells; reduce CSC frequency; delay tumor recurrence; | 74 | EudraCT No: 2014-001175-3 |
| GD16-PTX-NP | Nanomedicine | DLL4 | Pharyngeal squamous cancer | Inhibit tumor growth and angiogenesis | 166 | None |

Abbreviations: CSC, cancer stem cell; HUVECs, human umbilical vein endothelial cells.

positive effects of miRNA-34a include its inhibitory effects on the stem cell-like self-renewal, invasion and migration of tumor cells induced by DLL1/Notch signaling.^{51,151,152} However, miRNA-34a also reverses the anti-tumorigenic functions of DLL1/Notch signaling, inducing the trans-differentiation and chemoresistance of tumor cells.^{53,153} In addition, due to the different effects of DLL3 on Notch signaling and cancer progression, two DLL3 antagonists miRNA-518d and miRNA-18 have been shown as anti-tumorigenic and tumorigenic, respectively.^{58,154} This highlights the need to accurately define the functions of DLL prior to the use of miRNA-targeting strategies.

Antibodies

Humanized anti-DLL4 (anti-hDLL4) IgG antibodies are commonly used in pre- or clinical studies to inhibit tumorigenicity by reducing CSC frequency^{79,94,155,156} and to disrupt the tumor vasculature by inducing the production of immature non-functional blood vessels.^{79,132,136,137,157} However, despite its potent anti-tumor activity, anti-DLL4 antibodies show high levels of toxicity.¹⁵⁸ Upon the assessment of anti-hDLL4 IgG antibodies in athymic nude mice, anti-DLL4 antibodies led to nonlinear pharmacokinetics (PK) and rapidly distributed to several normal tissues including lung and liver. At doses greater than 10 mg/kg, its clearance decreased.¹⁵⁹ DLL4 blockade in normal tissues can remove the inhibition of VEGF-R2, which perturbs Notch/VEGF signaling, alters vascular homeostasis, and promotes the pathological activation of ECs.^{160,161} In the

livers of mice, rats and cynomolgus monkeys, anti-DLL4 treatment leads to pathological changes including sinusoidal dilatation (SD), centrilobular hepatic cord atrophy, bile ductular proliferation and abnormal liver function. In the skin, heart and lungs of rats, vascular lesions have also been observed.¹⁶⁰ The functional angiogenesis and growth of early tumors can be achieved by lower levels of DLL4, as observed in pre-cancerous skin papillomas.¹⁶¹ To reduce the DLL4 blockade-associated toxicity of full-length IgG, anti-DLL4 F(ab')₂ antibodies have been developed, which have a shorter half-life and are more rapidly cleared compared than anti-hDLL4 IgG antibodies, but maintaining robust anti-tumor activity.¹⁶² However, although general toxicity is reduced, the liver toxicity of anti-DLL4 F(ab')₂ at the genetic level is unavoidable.^{162,163} The anti-DLL4 nanobody 3Nb3, has a smaller size (15 KD) and higher affinity and specificity to the DLL4 antigen on the surface of tumor cells and ECs.^{164,165} As nanobodies generally have a reduced half-life and higher clearance compared to antibodies, they are speculated to reduce DLL4 blockade-associated toxicity, although this has not been confirmed experimentally. In addition to nanobodies, a DLL4-targeted nanomedicine GD16-PTX-NP has been developed and exhibits antiangiogenic effects on subcutaneous FaDu xenografts in pre-clinical studies.¹⁶⁶ As a nanoparticulate drug delivery systems (nano-DDS), GD16-PTX-NP controls the release and long-circulating features of the antiangiogenic model drug paclitaxel and is dependent on a peptide GD16 (H2N-GRCTNFHNFYICFPD-CONH2) to bind tumor endothelial DLL4.¹⁶⁶

DLL4 mediates tumor resistance to the VEGF inhibitor bevacizumab, leading to tumor regrowth and the formation of residual well-organized large vessels.^{68,141} The combined inhibition of DLL4 and VEGF signaling can re-sensitize tumors to VEGF inhibition, increasing tumor hypoxia and markedly reducing tumor growth and angiogenesis.^{141,167} Recently, several bispecific monoclonal antibodies targeting both hDLL4 and hVEGF have been established and exhibit inhibitory effects on tumor cells and the vasculature. Based on the different structural designs, anti-hDLL4 and anti-hVEGF bispecific antibodies include Navicixizumab (knob-in-hole),¹⁶⁸ HD-105 (scFv₂-Fc),¹⁶⁹ HB-32 (CrossMAB)⁸¹ and ABT-165 (DVD-Ig).⁸² Toxicity studies in cynomolgus monkeys indicate that ABT-165 lacks antidrug antibody responses and is well-tolerated at doses up to 200 mg/kg with non-adverse effects in the liver and thymus. This demonstrates more favorable pharmacokinetic and safety profiles than anti-hDLL4 IgG antibodies.⁸²

Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs) use antibody binding to specific antigens on the tumor cell surface to deliver small-molecule chemotherapeutic agents into the tumor cells to promote cell killing. Rovalpituzumab tesirine/SC16LD6.5/Rova-T is a DLL3-targeted ADC that contains an anti-hDLL3 IgG1 antibody, a linker and a pyrrolobenzodiazepine dimer toxin.³⁶ In pre-clinical studies, Rova-T effectively targeted DLL3 and inhibited tumor growth in “patient-derived xenograft” mouse models of neuroendocrine tumors including SCBC, NEPC, SCLC and LCNEC.^{28,111,113} In a phase I clinical study (NCT01901653) of 72 SCLC and 8 LCNEC USA patients, Rova-T produced an improved overall response (objective response rate (ORR) 38%) in DLL3-high patients ($\geq 50\%$) compared to patients with low DLL3 expression ($< 50\%$) (ORR 0%).¹⁷⁰ In a recent phase I clinical study (NCT03086239) of 63 Japanese SCLC patients treated with Rova-T, 17% with DLL3-high expression ($\geq 75\%$) had an objective response, with 56% achieving disease control.¹⁷¹ In addition, the median overall survival of patients with DLL3-high expression (7.4 months) was longer compared to those with DLL3-low expression (5.1 months).¹⁷¹ In a single-arm, Phase II TRINITY trial (NCT02674568) which assessed the safety and efficacy in a third-line treatment setting for 261 patients with SCLC, Rova-T treatment showed modest anti-tumor effects.¹⁷² ORR was 16% in the DLL3-high

expression ($\geq 75\%$) group and the disease control rate (DCR) was 24%. Unfortunately, grade 3–5 treatment-emergent adverse event (TEAE) occurred in 213 (63%) patients and drug-related serious TEAEs occurred in 100 (30%) patients. The most common TEAE was fatigue (38%) (n=130 patients), with the most common drug-related serious TEAE being photosensitivity reactions (42%) (n=143 patients).¹⁷² The TAHOE Phase III trial NCT03061812 compared Rova-T to topotecan as second-line therapy for SCLC, but was recently halted due to a shorter OS in the Rova-T arm. An additional MERU phase III trial NCT03033511 evaluated the efficacy of Rova-T as a first-line maintenance therapy following first-line platinum-based chemotherapy for advanced SCLC. However, the trial has been terminated as Rova-T treatment had no survival benefit at a pre-planned interim analysis. The clinical results suggest that the exploration and modification of the dose and schedule of DLL3-targeted drugs for SCLC treatment are necessary to reduce toxicity and improve efficacy. Despite the cessation of phase II and III trials, DLL3-expressing tumor cells remain a high-value target, and an additional phase I clinical trial NCT03000257 is recruiting participants to evaluate the safety and tolerability of Rova-T in combination with ABBV-181 for advanced solid tumors.

MvM03 and MGD03 are two novel DLL4-targeting ADCs containing an anti-hDLL4 IgG2a antibody MMGZ01, a linker, and a cytotoxic agent (MMAE and Doxorubicin respectively). In vitro and in vivo experiments in breast cancer models highlight the potent anti-tumor activity of MvM03 and MGD03, suggesting the potential therapeutic value of anti-DLL4 ADCs.¹⁷³

Conclusion

When the Notch receptor is activated, NICD is released following proteolytic cleavage, and DLLs determine the activation of downstream signaling pathways that can be oncogenic or tumor-suppressive. DLLs act as ligands at the cell surface, cytoplasm and nucleus, regulating the behavior of tumor cells, tumor EC cells and tumor-infiltrating immune cells.

Membrane DLL1 expression in cancer cells plays an oncogenic role through its activation of Notch signaling. However, active DLL1 can prevent tumor neovascularization and is critical for anti-tumor T cell immunity in the tumor hematopoietic environment.^{21–23,53,108,109} DLL3 localizes to the plasma membrane of tumor cells and acts as a reliable biomarker to predict cancer progression and a poor clinical

outcome. Pre- and clinical trial results indicate that membrane DLL3 is a potential target for preventing tumor growth.^{28,113,170} DLL4 plays an oncogenic role by regulating cell behavior and tumor angiogenesis. DLL4 antagonizes VEGF-mediated neovascularization and promotes the formation of large, mature and well-perfused vessels, all of which are beneficial to tumor growth.^{68,118,134} However, a positive correlation between DLL4 and tumor angiogenesis were also identified, though the mechanisms are complicated.

To promote the tumor-suppressive functions of DLLs and to avoid oncogenicity, numerous DLL-targeting strategies have been proposed. These include (1) DLL mimics that can activate or inactivate Notch signaling, including DLL recombinant proteins; (2) DLL binding molecules that inhibit their activity, including anti-DLL antibodies, bispecific antibodies and nanobodies; (3) DLL binding molecules that deliver cytotoxic agents or recruit CD3-positive T cells to kill DLL-positive cells, including nanomedicines, ADCs, HLE BiTE[®] antibodies and CARs; (4) Methods to inhibit the expression of DLLs, including miRNAs and therapeutic peptides. (5) Blocking other oncogenic pathways that are activated by DLL/Notch signaling, such as NF- κ B, PI3K/Akt and MMP-2/9 signaling.

In conclusion, DLLs play complex roles in the regulation of cancer progression through a variety of pathways, but are not restricted to cell-surface Notch-signaling. Prior to the clinical application of DLL-targeted therapies, their precise roles in a range of cancers must be fully understood. In addition, DLL-targeted drugs exhibit non-negligible side effects, and their effectiveness and safety now require improvement.

Abbreviations

ADAM, A Disintegrin and metalloproteinase domain-containing protein; Hes, hairy enhance of split; Hey, Hairy/Enhancer of split related with YRPW motif; PDZ, Postsynaptic density protein 95 (PSD-95), Discs large (DLG), Zonula occludens 1 (ZO-1); PD-1, programmed cell death protein 1; BHLH, basic helix-loop-helix; PTEN, phosphatase and tensin homologue; Pofut1, protein o-fucosyltransferase 1; IDH, isocitrate dehydrogenase; Akt, protein kinase B; PI3K, phosphatidylinositol 3-kinase; NF- κ B, nuclear factor- κ -gene binding; MMP, matrix metalloproteinase.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81760328).

Disclosure

The authors declare that they have no competing interests.

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