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Review

Notch signaling: Emerging molecular targets for cancer therapy

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ABSTRACT

The Notch signaling pathway is a highly conserved developmental pathway, which plays a critical role in cell-fate decision, tissue patterning and morphogenesis. There is increasing evidence that this pathway is dysregulated in a variety of malignancies, and can behave as either an oncogene or a tumor suppressor depending upon cell context. This review highlights the current evidence for aberration of the Notch signaling pathway in a wide range of tumors from hematological cancers, such as leukemia and lymphoma through to skin, breast, lung, pancreas, colon and brain tumors. It proposes that the Notch signaling pathway may represent novel therapeutic targets and will be a welcome asset to the cancer therapeutic arena.

Contents

Abbreviations: ACL, adenocarcinoma of the lung; ADAM, a disintegrin and metalloprotease; AML, acute myeloid leukemia; APP, amyloid precursor protein; B-CLL, B-chronic lymphocytic leukemia; c-IAP2, cellular inhibitor of apoptosis protein 2; CSC, cancer stem cell; DBZ, dibenzazepine; DN-MAML1, dominant negative-mastermind-like 1; GBM, glioblastoma; GIT, gastrointestinal tract; GSI, γ-secretase inhibitor; HD, heterodimerization; hEGFRs, human epidermal growth factor receptors; Herp, Hes-related repressor protein; Hes, Hairy and E (spl); HIF, hypoxia-inducible-factor; LNX, ligand of Numb-protein X; MB, medulloblastoma; MMTV, mouse mammary tumor virus; NICD, Notch intracellular domain; NSCLC, non-small cell lung cancers; *O*-Fut, *O*-fucosyl transferase; PcG, polycomb group; PDAC, pancreatic ductal adenocarcinoma; PH, polyhomeotic; SCC, squamous cell carcinoma; T-ALL, T-cell acute lymphoblastic leukemia; TACE, TNF-α converting enzyme; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein.

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1. Notch signal transduction cascade

The Notch pathway is an evolutionally conserved signaling pathway that has been implicated in a wide variety of processes, including cell-fate determination, tissue patterning and morphogenesis, cell differentiation, proliferation and death. Notch families are single-pass transmembrane proteins that have dual functions as both cell surface receptors and nuclear transcriptional regulators. The Notch was initially discovered to be responsible for the specific phenotype displayed as 'notches' at the wing blades of *Drosophila melanogaster* [1]. The gene was cloned in 1985 [2]. In mammals, the Notch families have four receptors (Notch1–4). Each Notch receptor is synthesized as a full-length precursor protein (300–350 kDa) consisting of extracellular, transmembrane, and intracellular domains that correlate with different cellular functions. The unprocessed precursors are then cleaved at the S1 site by furin-like convertase within the Golgi apparatus and re-assembled as a heterodimer on the cell surface [1]. Notch ligands are also transmembrane proteins and there are five Notch ligands (Jagged 1-2, Delta-like (Dll) 1, 3 and 4) in mammals. Notch signaling activation is initiated by ligand–receptor binding between two adjacent cells. This interaction of the ligand–receptor induces a conformational change in Notch receptors that lead to two successive proteolytic cleavages in Notch receptors. The first cleavage is mediated by metalloprotease (ADAM17 ((a disintegrin and metalloprotease 17)/TACE (TNF-α converting enzyme))) at the extracellular domain (S2) [3,4]. This makes Notch susceptible to the second cleavage at the transmembrane domain (S3), which is carried by γ-secretase, a five-subunit complex. The γ-secretase complex is composed of presenilin1 and 2, nicastrin, Pen-2, and Aph1 [5,6]. Following these two cleavage steps, the Notch intracellular domain (NICD) is released to the cytoplasm, and enters into the nucleus to activate the transcription of Notch target genes. Following NICD translocation into the nucleus, NICD binds to a transcriptional repressor CSL (also known as CBF1, or RBP-Jκ) to displace the co-repressor complex. Binding with NICD switches CSL into an activated state. Additionally, NICD/CSL complex recruits co-activators, such as mastermind-like (MAML) [7], and p300 that facilitate the transcriptional activation of Notch target genes [8] (Fig. 1). Primary Notch target genes include two families of transcriptional factors, Hes (Hairy and E (spl)) and Herp (Hes-related repressor protein) (also known as Hey/Hesr/HRT/CHF/gridlock). The helix-loop-helix domain in both Hes and Herp families determines the dimerization of Hes and Herp proteins. Homo- or heterodimers of Hes and/or Herp bring about repression of transcription by interacting with other co-repressors or sequestering transcriptional activators [9]. Other Notch target genes include cyclins D1 [10], p21 [11], NF-κB [12], pre-Tα (pre-T-cell receptor alpha chain) [13], GATA3 [14], NRARP [15], c-Myc [16] and Deltex1 [17].

In addition to the canonical activation of the Notch pathway, there is increasing evidence that Notch can signal in CSL-independent modes [18]. For instance, Notch signaling promotes the maturation of both $CD4^+$ and $CD8^+$ single positive thymocytes through CSL-independent pathways [19]. Activation of CSL-dependent Notch signaling can prevent the differentiation of

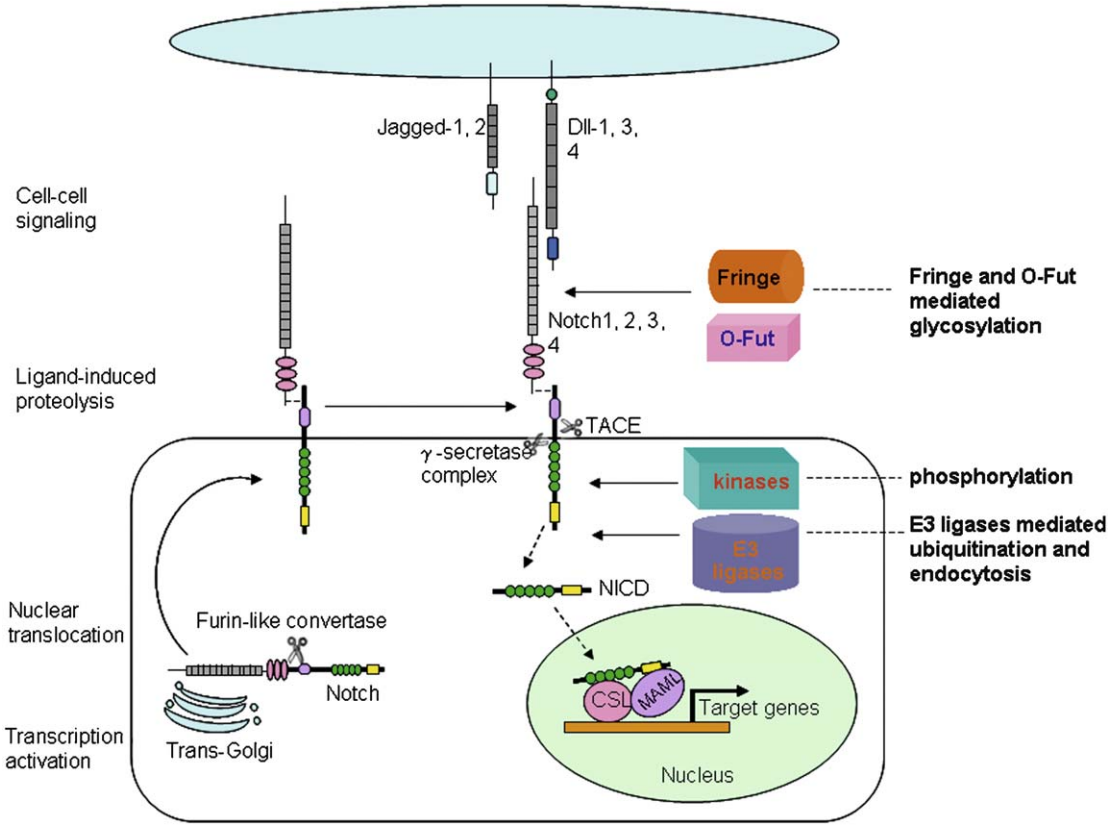

Fig. 1. The Notch signaling cascade. The Notch receptors (Notch1-4) are single-pass transmembrene proteins that are activated by the Delta-like and Jagged families of membrane-bound ligands expressed on adjacent cells. Upon furin-mediated trans-Golgi digestion, Notch proteins are transported to the plasma membrane and form matured heterodimer on the cell surface. Interaction with ligands leads to two additional proteolytic cleavages (TACE and γ-secretase complex) that liberate the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates to the nucleus, where it forms a complex with the DNA binding protein CSL. Co-activators, such as MAML, are recruited to the NICD–CSL complex, leading to the transcriptional activation of Notch target genes. Notch receptors can be post-translationally modulated by glycosylation, which are mediated by the enzymes of the glycosyltransferase Fringe and *O*-fucosyl transferase 1 (*O*-Fut), and phosphorylation. In addition, Notch can be regulated by different E3 ligases to undergo ubiquitination and subsequent proteolysis or endocytosis.

C2C12 cells upon serum withdrawal, and this is likely to occur by inhibiting the function of the muscle-specific transcription factor MyoD [20].

2. Enzymatic modulation of Notch pathway

One characteristic of Notch signaling is the involvement of multiple enzymatic modulations, which serve to regulate Notch signal transduction. Besides ligand-triggered, metalloprotease and γ-secretase-mediated proteolytic cleavages, and furin-mediated Notch maturation, Notch signaling can be regulated by four E3 ligases (Su(dx)/Itch, Sel-10, Neutralized, and LNX (ligand of Numb-protein X)) to undergo ubiquitination and subsequent proteolysis. Notch endocytosis by a different class of E3 (Nedd4) promotes the degradation of Notch whereby activation of the Notch signaling is attenuated/terminated [21–24]. LNX also can ubiquitinate the Numb, a Notch antagonist for degradation, which enhances/stabilizes the Notch pathway activation [25,26].

Moreover, Notch receptors are post-translationally modified by glycosylation [27] and phosphorylation [28], adding further complexity to the regulation of Notch signaling. The Notch receptors can be glycosylated extracellularly at the EGF-like repeats. Enzymes that process the extracellular post-translational modification include the glycosyltransferase Fringe and *O*-fucosyl transferase 1 (*O*-Fut). Fringe enzymes add N-acetyl-glucosamine to the O-linked fucose to inhibit the binding of Notch receptors to Jagged. In contrast, Fringe potentiates Delta-initiated Notch activation [29]. The mechanism underlying such a ligand-dependent regulatory effect remains unclear. The Notch protein is phosphorylated variably on serines of the cytoplasmic domain [30]. The phosphorylated NICD can preferentially associate with Su(H). Formation of NICD/Su(H) complex may determine the subcellular location of NICD [28]. The studies of Notch post-translational modification by enzymes provide both a direction for further elucidation of the mechanisms that regulate Notch activation, and a new paradigm for the role of enzymatic modifications in Notch-related diseases, especially cancers.

3. Notch signaling in cancer and angiogenesis

Notch signaling is one of the critical pathways in embryonic development and patterning. Given that tumorigenesis and organ development are believed to share similar mechanisms, it is not surprising that developmental pathways, such as Notch, Wnt, and Hedgehog are employed by tumor cells for their development and progression. Highly aggressive tumor cells have been shown to carry many characteristics of embryonic progenitor cells and use the Notch signaling pathway to promote their survival. Dysregulation of the Notch pathway has been associated with a wide range of cancers [31–33]. The Notch pathway could be either oncogenic or tumor suppressive depending on the tissue and organ site in which it is expressed (Table 1). However, how does activation of a single pathway give rise to two opposite outcomes in different cell types and contexts remains to be a mystery. One explanation for this seemingly paradoxical response is that canonical Notch pathway turns on/off different tissue/cell-specific target gene(s) or downstream pathway(s) which determines ultimate effect of Notch signaling. For example, in keratinocytes, perhaps CSL only binds the *p21* promoter thereby Notch functions as a tumor suppressor in this type of cells. Another potential explanation is that it depends upon other cooperative signaling(s). For instance, Notch1-deficient mice develop spontaneous, highly vascularized basal-cell carcinoma (BCC)-like tumors. In both mouse and human, BCC is frequently associated with deregulated Hedgehog (Shh) signaling, and Notch1-deficiency in the mouse skin leads to increased Gli2 expression, which is a downstream component of the Shh pathway [34]. Another pathway that seems to be deregulated as a consequence of loss of Notch1 is Wnt pathway, which results in increased β-catenin-mediated signaling in hyper-proliferative skin and primary tumor lesions, suggesting that Notch might suppress Wnt signaling in the skin [34]. The cross-talk between these pathways comprehensively determines the identity and threshold of downstream pathway(s) which controls cell fate. With respect to the different roles of Notch in cancers, further studies are needed to specifically identify the underlying mechanisms.

The general mechanisms of deregulation of Notch signaling characterized in cancers include chromosomal translocation (t (7, 9)) resulted constitutive expression of NICD [35], gain-of-function mutations in Notch1 in human T-cell acute lymphoblastic leukemia (T-ALL) [36], gene amplification of Notch3 in ovarian serous carcinoma [37], and the low levels of the Notch antagonist Numb in human breast cancers [38]. One main difficulty in the Notch study is to address how this simple, direct pathway gives rise to two opposite effects in different cell types and contexts. This review recapitulates the recent studies about the multi-functions of Notch and the potential therapeutic implications in cancers.

4. Notch in hematological tumors

Notch activation has been implicated in tumorigenesis of various hematological diseases, including leukemias, lymphomas, and multiple myeloma. In 1991, it was discovered that the

Table 1
Involvement of aberrant Notch signaling in a wide variety of cancers. Notch signaling may act as a tumor suppressor or a promoter depending on the cell type and cell context.

Tumor type	Notch/ligand	Function	Reference
T-cell acute lymphoblastic leukemia (T-ALL)	Notch1	Oncogenic	[37]
Acute myeloid leukemia (AML)	Jagged1	Oncogenic	[45]
B-chronic lymphocytic leukemia (B-CLL)	Notch1, Notch2/Jagged1, Jagged2	Oncogenic	[46]
Diffuse large B-cell lymphoma	Notch2	Oncogenic	[48]
Marginal zone lymphoma	Notch2	Oncogenic	[49]
Multiple myeloma (MM)	Notch1, Notch2/Jagged1	Oncogenic	[50,52]
Precursor B-cell acute lymphoblastic leukemia (pre-B-ALL)	Notch1–4	Tumor suppressive	[51]
Cutaneous squamous cell carcinoma (SCC)	Notch1	Tumor suppressive	[56]
Melanoma	Notch1	Oncogenic	[33,57–59]
Breast cancer	Notch4, Notch1,	Oncogenic	[61,63,65]
Human breast cancer	Notch2	Tumor suppressive	[64]
Human breast cancer	Notch1/Jagged1	Oncogenic	[66]
Non-small cell lung cancer (NSCLC)	Notch3	Oncogenic	[70,71]
Adenocarcinoma of the lung (ACL, a type of NSCLC)	Notch1/Jagged1, Dll1, Dll4	Tumor suppressive	[72,73]
Small cell lung cancer (SCLC)	Notch1/2	Tumor suppressive	[74,75]
Colorectal cancer (CRC)	Notch1/Jagged1, Jagged2, Dll4	Oncogenic	[84–86]
Pancreatic cancer	Notch1, Notch3/Jagged2, Dll4	Oncogenic	[87–89,91]
Glioblastoma	Notch2	Oncogenic	[93]

chromosomal translocation (t (7, 9)) leads to constitutive activation of Notch1 in human T-ALL [35]. Afterwards, the gain-of-function mutations in Notch1 receptor located at heterodimerization (HD) domain-encoding locus (exons 26 and 27), transcriptional activation domain and PEST domain (exon 34) [36] were identified as a novel mechanism for the constitutive activation of Notch1 in human T-ALL. Most Notch-dependent T-ALL cell lines and about 20% of primary T-ALL cell lines have mutations both in HD domains and PEST domains. When mutations occur at both sites in human T-ALL, they can produce synergistic effects in Notch activation [36]. c-Myc has been characterized to be a direct target of Notch1 in Notch-dependent T-ALL cell lines. Notch1 stimulates the transcription of *c-Myc* by binding to its promoter through a region containing a conserved CSL binding site [16]. In addition, stimulation of the mTOR pathway by mitogens requires concurrent Notch signals in T-ALL cell lines [39]. Interestingly, the effect of Notch1 withdrawal on the mTOR pathway can be rescued by enforced expression of c-Myc. This data indicates that c-Myc acts as an intermediary protein in between Notch and mTOR [39].

Although Notch activation represents a common feature in T-ALL pathogenesis, the role of Notch signaling in acute myeloid leukemia (AML) is not remarkable. Gain-of-function mutations of Notch have been seldomly established for AML [40,41]. Previous studies showed that even Notch1 activation remains low in primary AML cells, the Notch ligand Jagged1 is widely expressed [42,43]. A recent study indicates that the ligand stimulation of Jagged1 in primary AML cells from 12 patients has no effects on the self-renewal of AML cells, but instead promotes the differentiation of AML cells [44]. However, the underlying mechanism of how Notch signaling relates to the abnormal growth of AML remains unclear.

Notch receptors (Notch1 and Notch2) and their ligands (Jagged1 and Jagged2) are also constitutively expressed in B-chronic lymphocytic leukemia (B-CLL), but not normal B-cells. Moreover, Notch activation in B-CLL is accompanied by cellular inhibitor of apoptosis protein 2 (c-IAP2) and X-linked inhibitor of apoptosis protein (XIAP) expression. These represent additional novel potential therapeutic targets for the treatment of this disease [45].

Notch1 has been implicated in the determination of T-cell fate and the maturation of early T-cells in the thymus [46]. In contrast, Notch2 is widely expressed in mature B-cells and is indispensable for the development of marginal zone B-cell linage. In a recent study, five diffuse large B-cell lymphoma samples are found to harbor Notch2 mutations. These mutations are located on the PEST domain of Notch2, and confer increased activity to Notch2 receptors. This suggests that gain-of-function of Notch2 mutations plays a role in the oncogenesis of diffuse large B-cell lymphoma [47]. In addition, activating mutations in Notch2 are also involved in marginal zone lymphomas, another type of B-cell malignancy [48]. Although the mutations of Notch are not widely identified in B-cell tumors, high levels of active Notch receptors and ligands (Jagged1) have been reported in B-cell malignancy [47,49,50]. Collectively, these findings suggest a ligand-dependent Notch activation in B-cell tumors. Some studies demonstrate that activation of Notch signaling induces growth arrest and apoptosis in B-cell tumors including human B-cell leukemia, Hodgkin's disease, and multiple myeloma [50,51]. However, a number of studies provide opposite evidence concerning the role of Notch in B-cell malignancy, showing that active Notch actually promotes the proliferation of B-cell tumors [47,49,52]. To explain the discrepancy, further investigations and more meticulous examinations are necessary. Notch may exert different roles at different stages of B-cell development. It has been noted that Notch has an inhibitory effect during B progenitor commitment [53]. On the contrary, Notch may have positive effects on B-cell lineage during the later stage [54].

5. Notch in solid tumors

Deregulation of Notch pathway has been connected with the tumorigenesis in a variety of solid cancers. Notch signaling has been observed to have dual functions in skin cancers, depending on the cell type and context. As a consequence of loss of Notch1 activation in murine skin, basal-cell carcinoma-like tumors are developed, suggesting that the Notch pathway exerts tumor suppressive effects in the skin [34]. Inhibition of Notch signaling by dominant negative-MAML1 (DN-MAML1) in transgenic mice promotes the formation of cutaneous squamous cell carcinoma (SCC) and dysplastic precursor lesions, suggesting that the canonical Notch pathway confers epidermal skin cells a protection against cutaneous SCC [55]. A recent study shows that Notch1 promotes tumorigenesis of skin cancer by disrupting the skin-barrier integrity and producing a wound-like stromal microenvironment [56]. In contrast, evidence suggesting the tumorigenic activities of Notch1 signaling in melanoma has emerged. Notch1 has been shown to be activated in melanoma, and active Notch1 promotes progression of primary melanoma towards an advanced stage [33,57]. In addition, active Notch1 confers a transformed phenotype to primary melanocytes *in vitro* [58]. Recent findings further indicate that Notch1 signaling is indispensable for Akt and hypoxia to transform melanocytes, suggesting Notch1 is the downstream effector of Akt and hypoxia during melanomagenesis [59]. The molecular mechanism whereby the Notch signaling promotes melanoma progression has not been fully determined while previous studies revealed several potential downstream pathways, such as β-catenin pathway, Mel-CAM, N-Cadherin and MAPK pathway, which might mediate the oncogenic effect of the Notch signaling [33,57,58]. Further understanding of the precise role of Notch in specific skin cancers may help us develop a rationale for novel Notch-based therapeutics.

The tumorigenic activity of Notch in breast cancer has been established in mouse models. In 1987, the insertion of mouse mammary tumor virus (MMTV) into the *Notch4* locus, referred to as *int3* in the Czech II mouse strain was discovered [60], providing the first link between Notch and breast cancer. This group further reported that the MMTV-mediated insertion led to the truncated form of Notch4 protein, which is constitutively active. Besides Notch4, involvement of Notch1 in the formation of murine mammary tumors has also been identified. Notch1 is mutated by MMTV insertion and the truncated form of Notch1 functions as an oncogene in the development of mammary carcinomas [61]. Although the correlation of aberrant Notch signaling with mammary tumors is well established in murine models, such a correlation to human breast cancer is less robust. Callahan and his co-workers observed that expression of human-int3 (Notch4/In3) in transgenic mice blocked normal mammary development and induced the formation of breast tumors with an increased latency (average 18 months) [62]. However, in most studies regarding human breast cancers, activated Notch is only detectable at the protein level, rather than the mRNA level [63–65]. Parr's data further shows that Notch1 is increased in poorly-differentiated breast tumors, while high level of Notch2 is associated with a higher chance of survival, suggesting Notch1 exerts a tumor-promoting function and Notch2 functions as a tumor suppressor in human breast cancers [64]. So far, many studies have indicated that the Notch signaling plays an oncogenic role in breast cancers mainly through its interaction with other signaling pathways in mammary tumorigenesis. The well-characterized pathways which have the interactions with the Notch signaling during the oncogenesis of breast cancer include Ras, Erb2, TGF-β and Wnt signaling pathways. Four of seven cases of Notch1 positive human breast ductal carcinomas are H-Ras positive. This data suggests that Notch1 is the downstream effector of Ras signaling [66].

Furthermore, 80% of the mice with transgenic human Ras developed mammary tumors. Conversely, in mice with transgenic Ras and Notch inhibitor Deltex, only 20% developed mammary tumors. This highlights the cooperative functions of Ras and Notch in the development of breast cancers [66]. Of interest, tumors co-expressing high levels of Notch1 and Jagged1 correlate with poor survival of human breast cancers [65]. The human ErbB2 protein is a receptor tyrosine kinase that belongs to human epidermal growth factor receptors (hEGFRs) family [67]. The amplification and overexpression of the ErbB2 gene occurs in 20–30% of human breast cancers. ErbB2 behaves as an oncogene in collaboration with Notch1 in the development of mouse mammary tumors [61]. The overexpression of ErbB2 suppresses Notch activity and leads to decreased expression of canonical Notch target genes, including Hey1, Hes1, and Hes5 [68]. Furthermore, inhibition of ErbB2 by trastuzumab, a tyrosine kinase inhibitor (TKI), increases Notch1 activity and sensitizes the breast cancer to a GSI (γ-secretase inhibitor) [68]. This data suggests that combination of GSI with chemotherapy including trastuzumab may increase the efficacy of trastuzumab and reverse the resistance to ErbB2 targeted therapies.

In lung carcinomas, Notch signaling may behave as either an oncogene or a tumor suppressor depending on the tumor cell type. In non-small cell lung cancers (NSCLC), one study showed that Notch3 mediated signaling is active and promotes the growth of lung tumors [69]. In fact, inhibition of Notch3 by MRK-003, a GSI, reduces tumor cell proliferation and induces apoptosis in human NSCLC [70]. However, Chen et al. reported that Notch1 protein is downregulated in NSCLC cell lines and expression of constitutively active Notch1 in adenocarcinoma of the lung (ACL) cells causes cell death. These data suggests that the opposite functions of Notch signaling are highly context-dependent. Interestingly, under hypoxic conditions, Notch1 is dramatically upregulated, which seems to be essential for cell survival in ACL, a type of NSCLC [71]. These results indicate that oxygen concentration determines the biological effects of Notch1 signaling in ACL. A similar observation of the expression of Notch1–3 in the cell line A549 and SPC-A-1 of the human lung adenocarcinoma has also been obtained [72]. Overexpression of NICD inhibits the growth of the lung adenocarcinoma A549 cells *in vitro* by induction of cell cycle arrest and suppresses tumor growth of A549 in nude mice [72]. These findings suggest that the Notch signaling may function as a tumor suppressor in human lung adenocarcinoma cells. As a comparison, Notch1 and Notch2 have low-level expression in small cell lung cancers (SCLC), and overexpression of Notch causes growth inhibition in SCLC cells [73,74]. Furthermore, the Raf/MEK/MAPK pathway is activated by Notch signaling in SCLC [73]. This association may represent a possible cooperative interaction between the Notch and Ras signaling pathways in controlling SCLC cell growth.

Colorectal cancer is the second most common cause of malignancy deaths worldwide [75]. The early growth of colorectal tumors requires angiogenesis [76,77], which is dependent on the increased expression of pro-angiogenic factors (e.g., vascular endothelial cell growth factor-A (VEGF-A)) [77,78]. The Notch ligand, Dll4, is expressed by endothelial cells [79,80] and can be induced by VEGF [81] and hypoxia through hypoxia-inducible-factor (HIF)-1α [82]. A recent study has found that Dll4 is highly expressed in the endothelium of a large cohort of colon cancers and this expression is dramatically correlated with VEGF and hypoxia [83]. It implicates that Dll4-Notch pathway may be a potential therapeutic target of colon cancer. The Notch1 receptor has been discovered to be active in response to chemotherapy in colon cancer cells [84]. Down-regulation of Notch1 signaling with GSI sensitizes colon cancer cells to chemotherapy, whereas over-expression of NICD increases resistance to chemotherapy. Therefore, suppression of Notch1 signaling may be a novel therapeutic target to increase the sensitization of colon cancer cells to chemotherapy [84]. Reedijk et al. have suggested that expression of Jagged ligands and Notch1 as well as Notch receptor activation are constant features of human colon cancers, thus application of GSIs and other anti-Notch therapeutics may benefit patients with this disease [85].

Pancreatic cancer is one of the most aggressive human malignancies. Aberrant activation of the Notch pathway is commonly observed in pancreatic cancer [31,86–88]. High level expression of Notch ligands, including Jagged2 and Dll4, is detectable in the majority of pancreatic cancer cell lines. Inhibition of Notch pathway either by siRNA targeting Notch1 or by means of GSI (GSI18) alleviates anchorage-independent growth in PANC-1 cells, indicating that sustained Notch activation is required for pancreatic cancer maintenance [31,88]. Similarly, a recent study has showed that inhibition of γ-secretase activity by GSI reduces the growth of pre-malignant pancreatic duct-derived cells in a Notch-dependent manner and tumor development in a murine model of pancreatic ductal adenocarcinoma (PDAC) (K-Ras, p53 L/+ mice). These data suggests that Notch pathway is essential for PDAC progression. Interestingly, TW-37, a small molecule of Bcl2 family proteins, is able to inhibit cell growth and induce apoptosis in pancreatic cancer through a down-regulation of the Notch1 activity [89]. This finding suggests that the anti-tumor agent of TW-37 plays an inhibitory role in pancreatic tumor growth, at least, partially through the inactivation of Notch signaling. Suppression of Notch3 by Notch3-specific siRNA can increase gemcitabine-induced caspase-mediated apoptosis in pancreatic cancer through inactivation of PI3K/Akt-dependent pathway, suggesting Notch3 is a potential therapeutic target for pancreatic cancer [90].

Glioblastoma (GBM) is the most common malignant brain tumors in adults. Despite recent advances in surgery, imaging, chemotherapy and radiotherapy, outcomes in GBM remains poor and recurrence remains high. Therefore, novel efficient strategies are desperately needed to treat this disease. One recent study has showed that inhibition of Notch by GSIs or shRNA sensitizes glioma stem cells to radiation at clinically relevant doses. Such results suggest that integrated Notch signaling is involved in radio-resistance of glioma stem cells [91]. Another similar study demonstrates that Notch2 activation in GBM neurospheres increases their growth *in vitro* and Notch blockade with GSIs depletes the stem-like cells required for GBM *in vivo* and *in vitro* [92]. This data suggests that GSIs might be applied as useful chemotherapeutic agents by targeting cancer stem cells in gliomas. Hence, a combination regimen of GSIs and radiotherapy may be a highly efficacious strategy in the treatment of malignant GBM.

6. Notch in tumor angiogenesis

Neoplastic angiogenesis is one of the requirements for tumor growth and metastasis [93], as tumor greater than one cubic centimeter must develop its own blood supply to avoid necrosis. VEGF plays a key role in tumor angiogenesis, as does other pathways, including Notch [94]. Both Dll4 and VEGF are known as genes where loss of a single allele leads to embryonic lethality due to disrupted vascular hierarchy [95–97]. In mammals, many studies have demonstrated that Dll4 is induced by VEGF in tumor vasculature and functions downstream of VEGF to inhibit the VEGF-induced vessel growth, forming a negative feedback loop to inactivate VEGF [98,99]. It suggests that VEGF-induced Dll4 negatively inhibits tumor angiogenesis. However, recent studies, in fact, have shown that blockade of the Dll4-Notch pathway in mice induces tumor angiogenesis. Inhibition of Dll4 delays tumor growth [100–102]. This paradoxical phenomenon could be

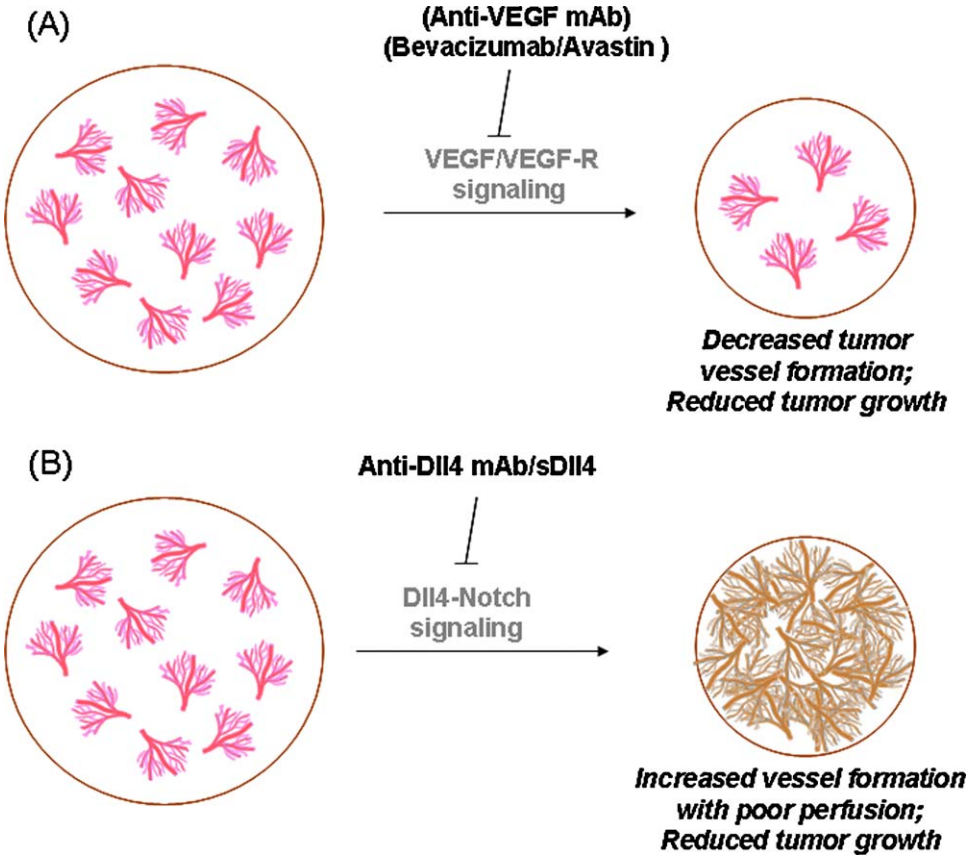

Fig. 2. Two anti-tumor angiogenesis models. (A) Neutralizing VEGF-VEGFR signaling by anti-VEGF monoclonal antibodies (bevacizumab/avastin) inhibits tumor vessel formation and reduces tumor size. (B) Antagonizing Dll4-Notch signaling by either anti-Dll4 antibodies or soluble Dll4 paradoxically promotes blood vessel formation but inhibits tumor growth. Reduced tumor growth is resulted from poor perfusion of newly formed capillaries.

explained by analyzing the functionality of blood vessels. The microvasculature formed from the enhanced tumor angiogenesis has poor integrity and perfuses the tumor poorly, thereby increasing hypoxia in tumors. In other words, Dll4 blockade causes the formation of nonfunctional vasculature, brings about a delay in tumor growth [100–102] (Fig. 2). Therefore, Dll4 has become a potential anti-angiogenic therapeutic target. Moreover, when combined with anti-VEGF treatment, Dll4 blockade is even more efficient in controlling tumor growth [101]. Concordantly, Li et al. have illustrated that Dll4 expressed in tumor cells activates Notch pathway in mice endothelial cells and improves tumor vascular function [103].

There are two advantages with respect to anti-Dll4 tumor therapy. First, the viability of treated animals is not compromised by administration of anti-Dll4 antibodies or soluble Dll4 ligand. Second, unlike the GSI, treatment with anti-Dll4 antibodies has no observable side effects on homeostasis in mice small intestine [102]. Due to the implication of Dll4-Notch pathway in immunity [104–106], further studies are needed to determine whether these anti-Dll4 therapies have non-angiogenic effects. Moreover, since hypoxia is induced in response to inhibition of Dll4 in tumors [101], additional investigations about the combination of anti-Dll4 treatment and other therapies are necessary.

7. Notch-targeted cancer therapeutics

A growing body of research and clinical evidence are in support of Notch's oncogenic or tumor suppressive role in a wide variety of cancers. It, therefore, places Notch signaling as a potential target for cancer therapeutics. An extensive understanding of Notch signaling cascade and its interaction with other pathways has provided us with insightful information for the identification of molecular targets to design effective therapeutic strategies (Fig. 3).

8. GSI therapy

Aberrant Notch signaling has been extensively linked to cancer and tumorigenesis. Ligand binding to the extracellular domain of the Notch receptor triggers intramembranous cleavage of the Notch receptor, carried out by the γ-secretase complex, resulting in cytoplasmic release of the NICD [5]. Therefore, blocking transmembranous proteolytic cleavage of Notch by GSIs could be a promising strategy for Notch-targeted therapeutics. The strategy inhibits NICD production, thus suppressing the downstream transcriptional events.

Over the past decades, synthetic GSIs have been successful in treating Alzheimer's disease, where defective γ-secretase cleavage of the substrate molecule amyloid precursor protein (APP) generates an Aβ42 variant of Aβ40 peptides, consequently resulting in plaque formation [107]. Since the proteolytic processes in Notch signaling activation are comparable with the processes involved in APP cleavage, GSIs are also capable of inhibiting the activation of Notch receptor, which offers an attractive targeted therapy for tumors dependent on aberrant Notch activity. It has been reported that treatment of T-ALL with GSIs including compound E, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-sphenylglycine-t-butyl ester), MRK-003 and YO01027 induces cell cycle arrest and apoptosis [36,108–110]. Treatment of medulloblastoma (MB) in a xenograft mice model with dipeptide GSI, DAPT, leads to decreased cell proliferation and increased apoptosis,

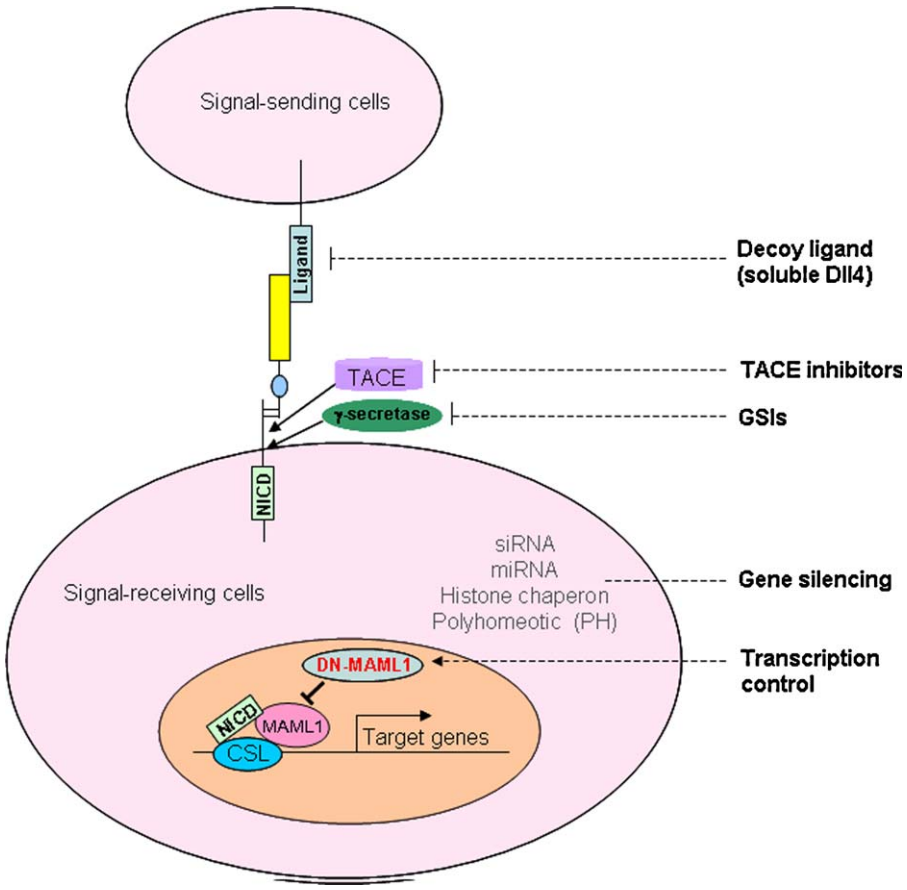

Fig. 3. Potential cancer therapeutics by targeting Notch signaling. These include decoy Notch ligand (soluble Dll4), disruption of two proteolytic cleavages by TACE inhibitor and GSIs, gene silencing by siRNAs, miRNAs, histone chaperon and polyhomeotic (PH) techniques, and transcriptional regulation (DN-MAML1).

suggesting that Notch activation contributes to human MB proliferation and survival [111]. Studies using synthetic GSI, dibenzazepine (DBZ), lead to the conversion of proliferative crypt cells into post-mitotic goblet cells in Apc$^{-/-}$ mice, suggesting GSIs might be of therapeutic benefit in colorectal cancer [112]. It has been noted that GSI1 suppresses breast cancer cell survival by promoting a cell cycle arrest at G2/M, which further triggers apoptosis [113]. Similarly, GSI-XII induces apoptosis of myeloma cells. Moreover, GSI-XII dramatically improves the sensitivity of myeloma cells to chemotherapeutic drugs such as doxorubicin and maphalan, representing a promising strategy for therapeutic intervention in multiple myeloma [114]. RO4929097 is a newly developed GSI with high selectivity and efficacy. This potent GSI has been proven to have an *in vitro* γ-secretase inhibitory activity. Of note, RO4929097 produces a less transformed, slow growing phenotype, rather than inhibiting tumor cell proliferation or inducing apoptosis. RO4929097 is active following oral administration and currently being tested in a phase I multidose escalation in patients with solid tumors [115]. The phase I study of another GSI, MK0752 for patients with advanced breast cancer is ongoing (http://clinicaltrials.gov/ct2/show/NCT00106145). Moreover, the exploratory study of MK0752 in combination with tamoxifen or letrozole to treat early stage breast cancer is currently under way. In addition, PF-03084014 has been tested in a phase I dose escalating study to determine its safety in patients with advance solid tumors and T-ALL (http://clinicaltrials.gov/ct2/show/NCT00878189).

While solid tumors have responded favorably to GSI, the majority of human T-ALL cell lines are not susceptible to these treatments. The molecular basis of GSIs resistance in T-ALL remains to be clarified. One recent study suggests that FBW7 mutations produce dominant-negative FBW7 alleles and confer GSI resistance in T-ALL cells [116]. Another study indicates that mutations on PTEN (a tumor suppressor) confer resistance to GSI therapy in human T-ALL cells. Loss of PTEN and constitutive activation of AKT in GSI resistant T-ALL cells increase glucose metabolism and bypass the requirement of Notch1 signaling to sustain cell growth [117,118]. In some human T-ALL cells, represented by CEM and Jurkat J6, when combined with chemotherapy drugs, GSI (compound E) antagonizes the effect of chemotherapy by decreasing apoptosis. Compound E also induces the expression of anti-apoptotic gene Bcl-xl mRNA and protein in CEM and Jurkat J6 cells [119].

The studies summarized above demonstrate a potential clinical application of GSI in anti-tumor therapy. However, one of the challenges is the inhibitor-associated side effects, especially cytotoxicity in the gastrointestinal tract (GIT) (Barten et al. [122]). For example, inhibition of Notch by GSI reverses glucocorticoid resistance in T-ALL and glucocorticoid treatment antagonizes the effects of Notch inhibition in the intestinal epithelium and protects from GSI-induced gut toxicity. Thus, combination therapies of GSIs and glucocorticoid can enhance the therapeutic efficacy in human T-ALL [120]. Advantages of GSI treatments include ease of administration, low cost, and oral bioavailability. In addition, it can block the activation of all four Notch receptors. However, unselectively blocking of all Notch homologues could also be disadvantageous since Notch proteins may have opposite effects in some tumors [116]. Furthermore, such compounds cause significant toxicities following chronic oral administration [121,122] and acquire resistance [116,117]. An-

other disadvantage is that since γ-secretase has a wide variety of targets other than Notch receptors, GSIs indiscriminately inhibit many signaling pathways [123]. Shelton et al. have recently developed a di-coumarin family of inhibitors that selectively inhibit APP cleavage by γ-secretase. They have revealed that the di-coumarin compounds induce a conformational change of γ-secretase by binding to an allosteric site that causes selective inhibition of Aβ42. This class of allosteric inhibitors provides the basis for development of Alzheimer disease therapeutic agents [124]. It follows that a broad number of drugs with sufficient specificity and affinity for inhibition of Notch receptor cleavage could be discovered for cancer therapy.

9. Other therapeutic approaches to Notch signaling inhibition

In addition to interfering with the cleavage of Notch receptors using GSIs, Notch ligand can be targeted using the more specific monoclonal antibodies (mAbs). mAbs selectively targeting Dll4 have been demonstrated to inhibit Notch signaling in endothelial cells and cause defective endothelial cell differentiation [102]. Furthermore, neutralizing Dll4 with a Dll4-selective antibody dysregulates tumor angiogenesis and inhibits tumor growth [101,102]. Remarkably, the combination of anti-human Dll4 and anti-mouse Dll4 results in additive anti-tumor activity in colon tumors [125]. In a NOD/SCID mice model of human colon cancer, administration of anti-Dll4 inhibits tumor growth and reduces cancer stem cell (CSC) frequency, indicating CSC might be the target for this drug [125]. Conversely, some mAbs have been implicated to specifically induce proteolytic cleavages in Notch3 [126]. The activating antibody (256A-13) binds to overlapping epitopes on one face of Notch3 and mimics certain effects of ligand-induced Notch activation [126]. These observations suggest that it is possible to develop antibodies that selectively modulate the activities of individual Notch receptors. The Notch-specific structural domain is the key towards the design of specific mAbs for Notch receptors. Currently, these mAbs are being developed and characterized as anti-angiogenic therapeutic agents [79,101,127].

Modulation of Notch signaling by other pathway components has also come to light. Notch1 has been shown to be induced by PI3K/Akt pathway in human arterial endothelial cells [81] and in melanoma development [59]. GSK3-α/β acted as negative regulators of Notch1 [128], and Notch2 was downregulated by GSK3β [129]. Phyllopod, a transcriptional target of the EGFR pathway can block Notch signaling pathway [130]. Inhibition of these pathways may indirectly modulate Notch signaling under certain circumstances.

microRNAs (miRNAs) are small (19–22 nts) noncoding regulatory RNA molecules that regulate diverse cellular processes [131]. Various miRNAs regulate the Notch pathway by binding to the 3′-untranslated region (3′-UTR) of Notch target mRNA. miRNA-34a has been found to be deregulated in human gliomas and forced miRNA-34a expression inhibits *in vivo* brain tumor growth by targeting multiple oncogenes (c-Met, Notch1 and Notch2) [132]. miRNA-34a molecule can also inhibit human pancreatic cancer stem cell renewal potential via the direct modulation of the downstream effectors of Notch1/2 and Bcl2 [133]. These studies suggest that restoration of tumor suppressor miRNA34 may provide a promising therapy for human gliomas and pancreatic cancers. In the screening of metastatic MB cell lines, miRNA 199b-5p has been observed to be a modulator of Notch signaling via its targeting of Hes1. Down-regulation of Hes1 expression negatively regulates the proliferation rate and anchorage-independent growth of MB cells [134]. Small interfering RNA (siRNA) is another type of RNA interference that has been used to inhibit Notch pathway activation [90,135,136]. Theoretically, any Notch pathway components can be targeted by specific siRNA. Thus, siRNAs-and/or miRNAs-mediated gene targeting approaches hold significant promise as potential anti-cancer therapeutic agents.

Evidence that histone chaperons play a diverse function during chromatin transactions is emerging [137,138]. ASF1, one of the H3/H4 chaperons, has been found to be required for repression of E(spl) Notch target genes through interactions with the Su(H)/H DNA binding complexes in *Drosophila*. These findings reveal that histone chaperons can act as gene regulators in silencing Notch-targeted genes [139]. However, the molecular mechanism by which ASF1 achieves gene silencing has yet to be delineated. A recent study demonstrates that the histone chaperons ASF1 and NAP1 facilitate removal of histone marks by two silencing complexes, LAF and RLAF, respectively, in different manners during Notch silencing [140]. Modulation of histone chaperons involved in the Notch pathway silencing might be a useful strategy in disease therapeutics.

PcG (polycomb group) gene encodes another epigenetic factor that silences Notch target genes. PcG proteins are involved in many physiological processes, including repression of homeotic gene transcription and modulation of cell proliferation [141]. A mutation in the gene locus (ph) encoding the PcG protein polyhomeotic (PH) induces cell proliferation [142]. In conjugation with Ras protein, these cells promote metastasis. PcG proteins are found to bind to many genes in the Notch pathway and control their transcription. When Notch is inhibited by either RNA interference or a dominant-negative form of the Notch pathway components, the over-proliferative phenotype of ph mutant cells could be reversed. It suggests that PH protein acts as a tumor suppressor in controlling cell proliferation by silencing Notch pathway components.

It is also possible to deregulate Notch pathway at the post-translational level by inhibiting the ubiquitination of Notch ligands for endocytosis [143,144] or blocking the fucosylation of Notch receptors [145,146].

Interestingly, a recent study shows that ADAM10/Kuz metalloprotease, but not ADAM17/TACE, is the main protease responsible for Notch1 cleavage at site 2 (S2) upon DSL ligand binding under physiological conditions in mouse fibroblast cells. However, ADAM10 is not required for ligand-independent cleavage of Notch1 receptors harboring gain-of-function T-ALL mutations [147]. Consistently, some other studies report that the ADAM requirement for Notch receptor activation is cell context-dependent. Specifically, ADAM10/Kuz is absolutely required for ligand-induced Notch activation, while Notch signaling independent of ligands requires ADAM17/TACE [148,149]. Identification of new drugs targeting the rate-limiting S2 cleavage may prove to be an interesting strategy to be exploited.

A DN-MAML1 at the length of 13–74 residues has been demonstrated to antagonize Notch signaling and cell proliferation in T-ALL cell lines [150,151]. This DN-MAML1 forms a structure of α-helix that binds to the extended groove formed by the assembly of NICD and CSL in human and *c. elegan* [152,153]. These data suggests that Notch transactivation complex (NICD–CSL–MAML1) might be a useful target for Notch inhibition by such α-helix like peptides. Recently, Moellering et al. have prepared peptide segments of the MAML1 binding site, and constrained them into α-helical conformation by hydrocarbon 'staples'. They reason that the stapled peptides bind to the CSL–NICD complex, preventing full-length MAML1 from binding and thereby directly inhibiting the transcription of Notch-targeted genes [154,155].

10. Perspectives

Accumulating evidence has emerged over the past decade that strongly supports the hypothesis that Notch signaling is one of the

most promising novel therapeutic targets in cancer treatment. Improved strategies for the clinical application of Notch pathway targeted therapies will need to consider: (i) specificity. Four Notch receptors may have distinct, even opposite, effects depending on cell context and tumor types. Notch2 is oncogenic in embryonal brain tumor growth while Notch1 inhibits the tumor growth [156]. Notch1 and Notch3 have overlapping functions in inducing murine mammary tumor phenotypes [157]. Two key Notch ligands, Jagged1 and Dll4 have been implicated in tumor angiogenesis. However, these two Notch signaling components regulate tumor angiogenesis by diverse mechanisms. Inhibition of Dll4 paradoxically induces increased tumor angiogenesis but reduced tumor growth, because newly growing tumor vessels are not functional with poor perfusion capacity. In contrast, Jagged1 expression in tumor cells promotes the growth of tumor vessels, suggesting a pro-angiogenic role of Jagged1 in tumors [158]. Hence, new classes of specific Notch inhibitory molecules, such as novel GSI compounds that are capable of selectively inhibiting specific Notch receptors, or specific anti-Notch inhibitory antibodies which could be engineered to block the specific member of Notch receptors in the tumor cells, need to be developed. Complete understanding of the mechanism of individual Notch ligand/receptor's function in different tumors will greatly increase our ability to improve the anti-cancer regimen under specific circumstances. In addition, identification of biomarkers to guide the selection of specific anti-Notch medicine or predict the response of various tumor cells to anti-Notch treatment will be a significant plus. (ii) Combined therapies. At present, it is difficult to achieve satisfactory therapeutic accomplishments with Notch-targeted monotherapy. Given the fact that Notch signaling interacts with many other pathways including PI3K/Akt, NF-κB and STAT3. Appropriate combination of Notch inhibitors with other individual medicines may prove to be synergistically beneficial in the clinical setting. Moreover, combined therapy will not only increase the anti-tumor effects of these drugs, but also improve their therapeutic window. (iii) Efficacy versus toxicity (side effect). In considering GSI-associated acute toxicity, the balance between efficacy and toxicity of GSI should be taken into account in future clinical applications. A new parenteral drug formulations aiming to avoid the toxic effects of GSI in the gut should be developed.

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