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Molecular Pathways: Translational and Therapeutic Implications of the Notch Signaling Pathway in Cancer

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Abstract

Over 100 years have passed since the first observation of the notched wing phenotype in *Drosophila melanogaster*, and significant progress has been made to characterize the role of the Notch receptor, its ligands, downstream targets, and crosstalk with other signaling pathways. The canonical Notch pathway with four Notch receptors (Notch1-4) and five ligands (DLL1, 3–4, Jagged 1–2) is an evolutionarily conserved cell signaling pathway that plays critical roles in cell-fate determination, differentiation, development, tissue patterning, cell proliferation, and death. In cancer, these roles have a critical impact on tumor behavior and response to therapy. Since the role of Notch remains tissue and context dependent, alterations within this pathway may lead to tumor suppressive or oncogenic phenotypes. Although no FDA approved therapies currently exist for the Notch pathway, multiple therapeutics (e.g., demcizumab, tarextumab, GSI MK0752, R04929097, and PF63084014) have been developed to target different aspects of this pathway for both hematologic and solid malignancies. Understanding the context-specific effects of the Notch pathway will be important for individualized therapies targeting this pathway.

Background

One hundred years ago, John S. Dexter observed serrations on the wing margin in *Drosophila melanogaster* (1). Decades later, Artavanis-Tsakonas and Young independently cloned the Notch receptor (2). The Notch signaling pathway is a highly conserved pathway among species (Fig. 1). In mammals, four type I transmembrane Notch receptors (Notch 1-4) are synthesized. After cleavage at the S1 site in the Golgi apparatus by a furin-like

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convertase, (3) it becomes glycosylated by O-fucosyltransferase (4, 5) and Fringe family N-acetylglucosaminidyl transferases (6); the processed heterodimers re-assemble on the cellular membrane (7). The extracellular subunits, of Notch1 and 2, both have 36 epidermal growth factor (EGF) repeats; Notch3 and Notch4 have 34 and 29 repeats, respectively, which correlate with affinity for their respective ligands (8). Additionally, the receptor contains a negative regulatory region comprised of three cysteine-rich Lin12/Notch repeats and a C-terminal region (9, 10). The other primary difference between the receptors rests within the transactivation domain (TAD) with either strong (Notch1), weak (Notch 2), or absent (Notch4) TAD (11). The Notch3 TAD is specific to activation of the *hes5* promoter (12).

Close proximity among cells within the microenvironment is required for ligand-receptor binding and interactions because the ligands remain immobilized as transmembrane proteins. Mammals have four distinct ligands (Jagged1-2, Delta-like [DLL] 1, 3, and 4). Distinct ligand affinities exist for the various receptors, altered by glycosylation, which influences downstream transcriptional activation. Activation of the pathway requires ligand-receptor binding; the ligand undergoes endocytosis within the ligand-emitting cell, which causes a mechanical disruption, changing conformation of the negative regulatory region, and susceptibility of the ectodomain to cleavage by ADAM17 metalloprotease/TNF- α converting enzyme (TACE) at site S2 (13, 14). A subsequent cleavage occurs within the TAD at S3 by presenilin- γ -secretase, liberating the intracellular domain of the Notch receptor (ICN) (15, 16). ICN forms a complex with the inactive DNA-binding factor CSL (CBF1/Suppressor of Hairless/Lag1) and recruits other co-activator proteins from the Mastermind-like family of proteins such as MAML1 (17, 18). The target genes activated by Notch depend on the cell type and ligand-receptor interaction at the cell surface. Frequent target genes include transcriptional repressors of the HES and HEY families, MYC, NF- κ B, cyclinD1, p21, CCND1/3, BCL2, pre-T α (pre-T-cell receptor alpha chain), GATA3, NRARP, Deltex1, and CCR7 (2, 19). Additional non-cognate ligands (e.g. EGFL7) (20) and soluble Jagged ligands have also been described (21).

Notch pathway in cancer

Expression of the four Notch receptors in adult and embryonic tissues varies widely with overlapping expression patterns, but they have unique roles during the generation of hematopoietic stem cells, T-cell and B-cell fate and lineage development, renal progenitor cells, and vascular morphogenesis (2, 22). Dysregulation of the Notch pathway has been implicated in a variety of hematologic and solid malignancies (2). Depending on expression patterns, the Notch pathway can be either oncogenic or tumor suppressive (Fig. 2), involved in either survival or death pathways, proliferation or growth arrest, or differentiation into terminally differentiated cells *versus* cancer cell “stemness” (23). Abnormal regulation of the Notch pathway may occur by a variety of mechanisms including mutational activation or inactivation, overexpression, post-translational modifications, and epigenetic regulation (2). In general, it seems suppressive in squamous cancers, but activating in hematological malignancies and adenocarcinomas, reflecting its normal functions in those tissues.

Notch as an oncoprotein

Notch1 is a well-characterized oncoprotein in T-cell acute lymphoblastic leukemia (T-ALL) and lymphomas; activating Notch1 mutations (either in the heterodimerization domain leading to a change in amino acid sequence causing ligand-independent metalloprotease cleavage at site S2 (24) or stop codon or frame shift mutations by deletion of the C-terminal PEST domain) are responsible for approximately 55–60% of T-ALL cases (25). Evidence for Notch as an oncoprotein in melanocytes (26), prostate (27) and breast tissue also exists (28, 29). Constitutively active Notch1 promotes melanoma cell growth, and the oncogenic effect of Notch1 on primary melanoma cells was mediated by beta-catenin (30). The MAPK and PI3K-AKT pathways are both activated in melanoma following Notch1 activation (31). Upregulated Notch signaling has been shown to be oncogenic for multiple hematologic and solid malignancies (2, 19, 32) (Fig. 2).

The mechanisms exploited by Notch for oncogenic effects include inhibition of apoptosis and induction of cellular proliferation. Within solid malignancies, activation of Notch can promote epithelial-to-mesenchymal transition. Anti-apoptotic effects may occur by Notch inhibiting the pro-apoptotic transcription factor, Nur77, upregulation of IAP, Bcl2, and FLIP. Increased proliferation may occur through enhanced CDK2, cyclin D1, and HES1 activity. Notch can suppress p53 expression, promote viability *via* the PI3K/AKT, ERK and NF κ B pathways, and protect against apoptosis *via* inhibition of JNK activation. Activated Notch can inhibit activity of Smad2-4, leading to decreased TGF β signaling (Fig. 1) (33).

Notch as a tumor suppressor

Notch has tumor suppressive roles in several malignancies, including skin cancers (Fig. 1). Notch1 knockout mice develop basal cell carcinomas and have increased Wnt and Hedgehog signaling (34). The mechanism of Notch as a tumor suppressor is less well understood, but may be related to inhibition of proliferation and induction of cell cycle arrest through upregulation of p21^{Cip1} and p27^{Kip1} and inhibition of β -catenin-mediated Wnt signaling (33).

The mechanisms by which Notch pathway can lead to tumor promoting or suppressive effects in different cell types are not fully understood. These mechanisms may include differential tissue and cell specific target genes, and varying cytokines and growth factors present in distinct microenvironments. For example, *p21*, a target gene in the keratinocytes in the epidermis may contribute to tumor suppressive effects because it regulates cell cycle progression (35). This may result from CSL only binding the *p21* promoter in certain tissue types, or crosstalk between Notch and *p62* (36).

Clinical-Translational Advances

Notch antibodies

Antibodies are most frequently targeted to the extracellular negative regulator region of the Notch receptor or the EGF repeats (37). Several Notch specific monoclonal antibodies against Notch1, Notch2, or Notch3 have been developed (38). In pre-clinical models, blocking Notch1 has been shown to decrease T-ALL tumor growth by inhibiting cancer cell

growth and by disrupting angiogenesis. However, dual Notch1 and 2 inhibition causes gastrointestinal side effects such as diarrhea (37, 39). A Phase I dose-escalation study of OMP-59R5 (humanized Notch2 and 3 blocking monoclonal antibody) in patients with solid tumors is ongoing (NCT01277146).

Alternative antibodies developed against this pathway target ligands (e.g., anti-DLL4 antibody and soluble DLL4-Fc fusion protein). The DLL4 antibody decreased tumor growth in multiple tumor models and caused defective cell fate differentiation. The role of DLL4 has been shown to be necessary for vascularization, but not vessel maintenance and DLL4 blockade promotes non-productive angiogenesis (40). Multiple early-stage clinical trials are ongoing with demcizumab, an anti-DLL4 monoclonal antibody (41). OMP-21M18 is being tested in combination with gemcitabine with or without Abraxane® (Celgene, Co., Summit, NJ, USA) in pancreatic cancer (NCT01189929), with FOLFIRI in colorectal cancer (NCT01189942), with carboplatin and pemetrexed in lung cancer (NCT01189968), and with paclitaxel in patients with platinum-resistant ovarian cancer (NCT01952249). Medi0639 is another anti-DLL4 antibody being evaluated in patients with advanced solid tumors in phase I trials (NCT01577745).

Notch decoys

Soluble decoys of Notch pathway receptors or ligands are highly potent therapeutics. Notch1, DLL1 and Jagged1 decoys (42) remain under current development. A Notch1 decoy reduced downstream signaling and led to decreased tumor angiogenesis with a 58% decrease in microvessel density (43). Monomeric and dimeric forms of DLL1 soluble decoys have been created by fusing the extracellular domain to either a series of myc epitopes or to the Fc portion of human IgG-1, respectively. Non-immobilized DLL1 inhibited downstream Notch functioning (44).

Gamma-secretase inhibitors (GSIs)

A wide variety of GSIs have entered clinical development. Because of the diversity of GSIs and their substrates, the targeting for Notch cleavage is often not highly specific. However, they have cytostatic and cytotoxic properties and act as competitive inhibitors of presenilin activity. RO4929097 is a GSI that pre-clinically showed decreased ICN expression *in vitro* and antitumor activity in multiple xenografts, with intermittent or daily dosing and prolonged efficacy (45). Tumor growth inhibition values ranged from 66% to 91%. Initial clinical testing suggested a favorable toxicity profile with primarily grade 1 or 2 toxicities of fatigue, thrombocytopenia, rash, chills, and anorexia (46). In a phase I study of patients with refractory metastatic or locally advanced solid tumors, tumor responses included one partial response in a patient with colorectal adenocarcinoma, one mixed response in a patient with sarcoma, and one complete FDG-PET response in a patient with melanoma (46). In a phase II study of 33 evaluable patients with metastatic colorectal cancer, there were no objective radiographic responses and six patients had stable disease. Development of RO4929097 has been discontinued (47). Another GSI, MRK-003, has been shown to induce cell cycle arrest and apoptosis by blocking Notch1 (48), with activity in T-ALL (49) and breast cancer (50). A related compound, MK-0752, which binds to presenilin 1, is being tested in multiple Phase I studies.

Other GSIs include PF-03084014, which has been tested in combination with chemotherapy in colorectal (51), breast (52), pancreatic (53), and triple-negative breast (TNBC) (54) cancer models, and with fludarabine in primary Notch1-mutated CLL (55). Clinical studies with PF-03084014 are ongoing in patients with leukemia, breast or pancreatic cancers. Although preclinical and early clinical activity is encouraging, limitations include non-selectivity and possible toxicities (56).

Inhibition of the Notch signaling pathway by GSIs has been shown to increase sensitivity of tumor cells to both cytotoxic chemotherapy and radiation (57, 58); however, the GSIs are not equally effective in combinations. For example, oxaliplatin activates Notch signaling, so while GSI34 sensitizes colon cancer cells to chemotherapy (59), MRK-003 decreases the apoptotic effect by at least 50% (60). This differential effect of GSIs combined with chemotherapy has also been seen in T-ALL where apoptosis is induced in some cell lines, but not in all (58). In TNBC, the combination of MK-0752 with docetaxel in preclinical and concurrent clinical studies decreased breast cancer stem cells (61). The mechanism for the synergistic activity of MK-0752 and PF-03084014 with docetaxel occurs because docetaxel activates the Notch pathway and suppresses NUMB, an endogenous Notch inhibitor (62).

Blocking peptides

Permeable peptides that interfere with the transcriptional nuclear complex represent attractive therapeutic options. A peptide that forms complexes with Notch1 and CSL, forms a transcriptionally inert nuclear complex, and inhibits the growth of Notch1-transformed T-ALLs (63). Another peptide, SAHM1, binds to the Notch1 and CSL complex and prevents MAML1 from binding (64). The clinical implication and pharmacodynamics of peptides still remain unknown.

There are two basic approaches to targeting the Notch pathway: one is blocking ligands or receptors with monoclonal antibodies and the other blocking downstream signaling with GSIs. Small molecule inhibitors tend to have more off target effects or inhibit multiple pathways, and this is typical with GSIs. Antibodies are more specific and tend to have longer duration of effect. The appropriate selection of therapy will depend on a greater understanding of the molecular pathways involved in a particular cancer. For example, mutations in Notch1 affecting its stability would be logical targets for blocking monoclonal antibodies. For targeting vasculature, DLL4, which is an endothelial specific Notch ligand, can be effectively targeted with a specific antibody.

Rational combination therapies

The Notch pathway interacts with other tumorigenic pathways including the PI3K/AKT, STAT3, and NF- κ B pathways. Combinations of GSIs with Hedgehog and Wnt inhibitors suppressed TALL growth *in vitro* (65). Both the Jagged1 and DLL4 ligands have been previously identified as key players in angiogenesis by separate mechanisms. Jagged1 can positively influence tumor angiogenesis by activating the Notch pathway on tumor endothelial cells (66). Rational combinations include targeting VEGF plus DLL4 since the latter can mediate resistance to anti-VEGF therapy. VEGF induces expression of DLL4 in endothelial and cancer cells *in vitro* under hypoxic conditions and activation of Notch by

immobilized DLL4 led to downregulation of VEGFR2 *via* promoter methylation. Blocking DLL4 upregulates VEGFR2, and the resultant increase in non-productive angiogenesis may be the result of endothelial cells becoming sensitized to VEGF signaling. This response represents a rational, biologic explanation for why targeting Notch ligands may be complementary to anti-VEGF therapy (67). Notch1 activity has been reported to be related to DNA repair pathways and resistance to DNA cross-linking agents in TNBC; a combination with DNA cross linking agents plus Notch1 inhibitors would be a logical approach (68–70).

In hormonally active malignancies such as ER α -positive breast cancers, combination therapy with GSI has shown promise in clinical trials (71). Combinations of exemestane and RO4929097 (72) and MK-0752 with tamoxifen or letrozole have led to partial responses and stable disease for some patients (73).

Targeting the Notch pathway *via* specific siRNA and/or miRNAs also remains a relatively untapped area that could hold future benefit in several malignancies. Notch1 has been identified as a direct target gene of miR-34a. Mir-34a has also been found to be downregulated in glioblastoma multiforme compared with normal brain tissues, and by targeting Notch1, tumor growth was inhibited (74). In pancreatic cancer, miR-34 may be involved in pancreatic cancer stem cell self-renewal, potentially *via* direct modulation of downstream targets Bcl-2 and Notch (75). Restoring miR-34 may provide future therapeutic benefit in brain and pancreatic malignancies.

Multiple strategies exist for targeting the Notch pathway in malignancies, but the most attractive therapies will target the specific Notch alteration within tumor types while avoiding Notch signaling in normal tissues. Overexpression of Notch receptors and/or ligands does not necessarily imply pathway activation, and pathway activation can lead to tumor suppressive or oncogenic effects. In these cases, simply providing an antibody, peptide, or decoy that targets the Notch receptor may be insufficient to decrease tumor growth or metastasis. Non-specific inhibition of the Notch pathway with GSI has been toxic. In a phase I study with RO4929097, several responses were noted, but further development has been discontinued.

Patient selection for therapy

As for many targeted therapies, selecting the correct patient is critical. Patients with activating mutations in their tumors seem to be obvious candidates for Notch therapies, but mutations in other genes (FBXW7) can affect Notch degradation. Also, the stroma affects Notch signaling in endothelial cells and does not require tumor mutation. Markers such as ICN, expression of ligands or early dynamic monitoring of response need to be integrated with early clinical studies.

Conclusions

Our understanding of the Notch pathway has progressed substantially over the past century from an observation of the notched wing phenotype in *Drosophila melanogaster* to the context and tissue-dependent roles of this signaling pathway in hematologic and solid

malignancies. No FDA approved Notch targeted therapies currently exist despite the progress in understanding this signaling pathway within different malignancies. GSIs have been the most broadly developed, but due to their lack of specificity, gastrointestinal side effects, and low response rates, further development may be challenging. More selective and potent inhibitors and select combinations with chemotherapy or other biologically targeted drugs should be pursued. Future important directions for this signaling pathway include:

1. To determine the roles the Notch pathway at different points in tumorigenesis, metastasis, and self-propagation of cancer stem cells
2. To develop biomarkers for sensitivity of cancers and stroma
3. To develop rational combination therapies.

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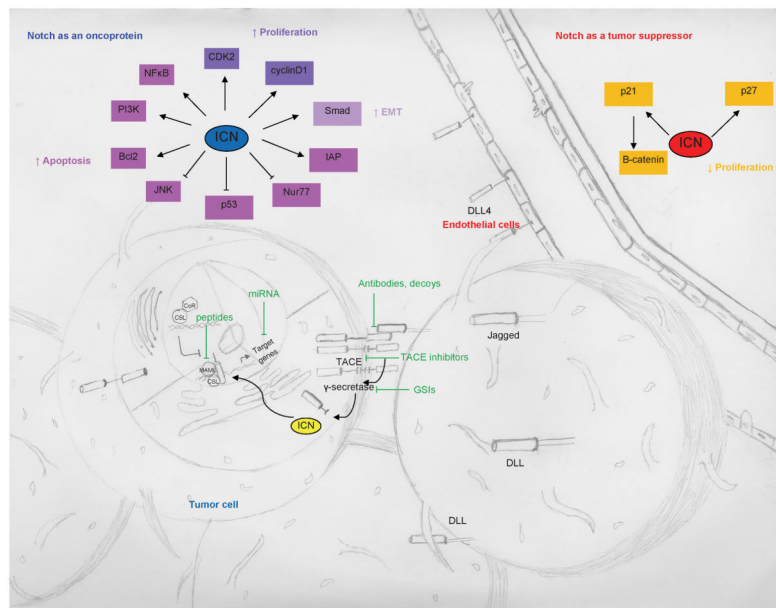


Figure 1.

Notch receptors (Notch1-4) and ligands (DLL1, 3 and 4, Jagged 1-2) are expressed in tumor, normal, and endothelial cells. After ligand binding, the ICN is generated after cleavage events by ADAM/TACE proteases and γ -secretase. The ICN travels into the nucleus, interacts with multiple transcriptional regulators including CSL, displaces CoR, and recruits MAML to activate transcription of target genes. Potential cancer therapeutics that target Notch signaling include antibodies, peptides, miRNAs, TACE inhibitors, and GSIs. Notch can function as a tumor suppressor or is oncogenic and activate/inhibit different downstream targets depending on the malignancy and microenvironment.

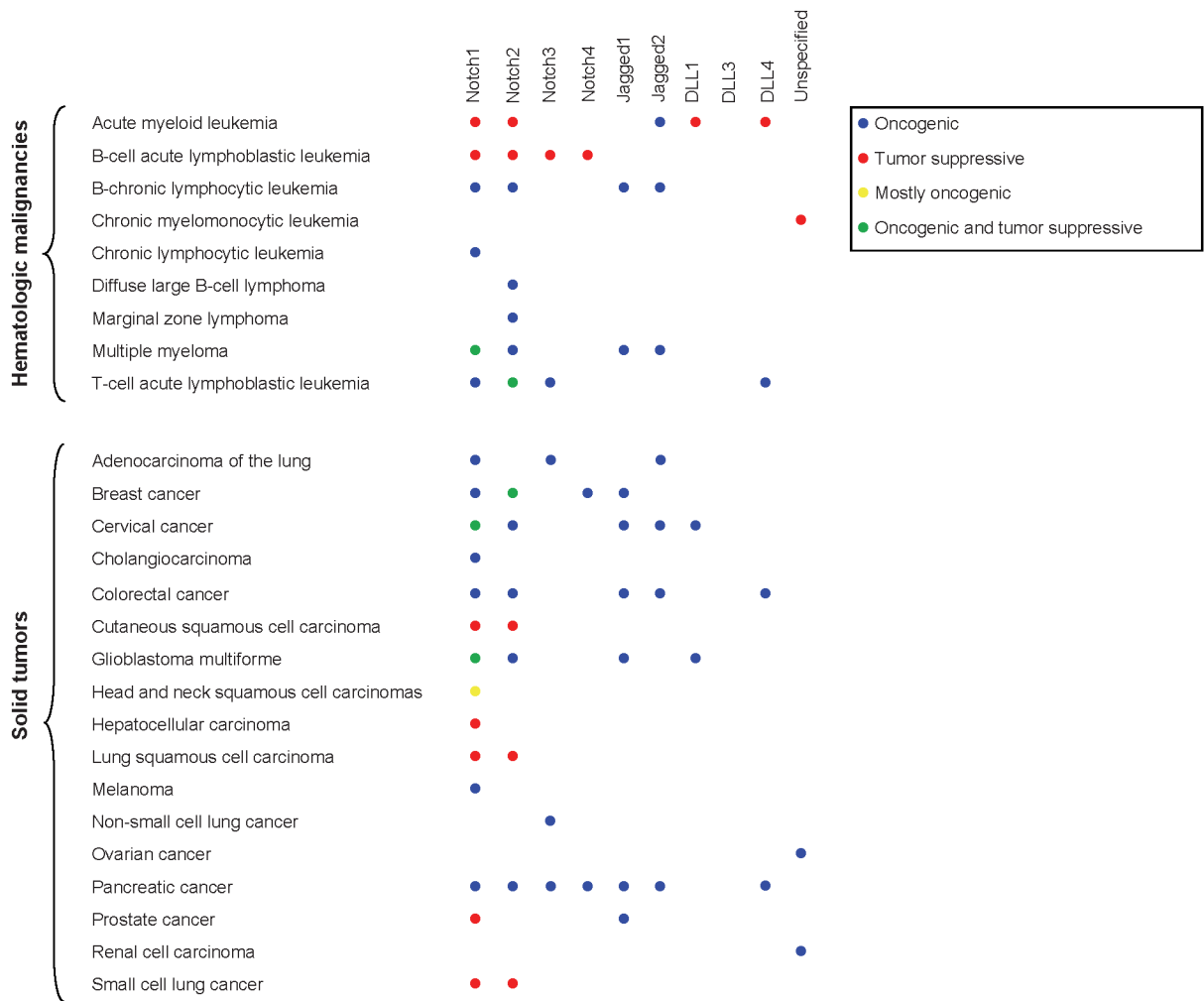


Figure 2. Aberrant Notch signaling occurs in a wide variety of solid and hematologic malignancies, and its role may be oncogenic or tumor suppressive depending on the tissue type and cellular context.