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 cross-talk 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. Because 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 MK-0752, 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 convertase, (3) it becomes glycosylated by O-fucosyltransferase (4, 5) and Fringe family N-acetylgalcosaminid transferases (6); the processed heterodimers reassemble on the cellular membrane (7). The extracellular subunits, of Notch1 and 2, both have 36 EGF repeats; Notch3 and Notch4 have 34 and 29 repeats, respectively, which correlate with affinity for their respective ligands (8). In addition, the receptor contains a negative regulatory region composed 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/TNFα 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; refs. 15, 16). ICN forms a complex with the inactive DNA-binding factor CSL (CBF1/Suppressor of Hairless/Lag1) and recruits other coactivator 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-kB, cyclinD1, p21, CCND1/3, BCL2, pre-Tα (pre-T-cell receptor α-chain), GATA3, NRARP, Deltex1, and CCR7 (2, 19). Additional noncognate 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

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doi: 10.1158/1078-0432.CCR-14-0809
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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, posttranslational modifications, and epigenetic regulation (2). In general, it seems suppressive in squamous cancers, but activating in hematologic 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% to 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 β-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. Antiapoptotic effects may occur by Notch inhibiting the proapoptotic 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-κB pathways, and protect against apoptosis via inhibition of JNK activation. Activated Notch can inhibit activity of Smad2-4, leading to decreased TGFβ 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 p21Cip1 and p27Kip1 and inhibition of β-catenin–mediated Wnt signaling (33).

The mechanisms by which Notch pathway can lead to tumour-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 cross-talk 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 preclinical 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 1 dose-escalation study of OMP-59R5 (humanized Notch2 and 3 blocking monoclonal antibody) in patients with solid tumors is ongoing (NCT012777146).

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 nonproductive 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.) 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 1 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. Nonimmobilized DLL1 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 1 studies.

Other GSIs include PF-03084014, which has been tested in combination with chemotheraphy in colorectal (51), breast (52), pancreatic (53), and triple-negative breast (INBC; 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 nonselectivity and possible toxicities (56).

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-kB pathways. Combinations of GSIs with Hedgehog and Wnt inhibitors suppressed T-ALL 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 because 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 nonproductive 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 ERα-positive breast cancers, combination therapy with GSIs 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 Bel-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. Nonspecific inhibition of the Notch pathway with GSIs 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 of 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: R.A. Previs, R.L. Coleman, A.L. Harris, A.K. Sood
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.L. Coleman
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.A. Previs, R.L. Coleman, A.K. Sood
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Grant Support
This work was supported, in part, by NIH grants (P50CA083639, CA109298, P50CA098258, U54CA151668, U2HT000943, CA016672, U54CA96300, and U54CA96297), the Cancer Prevention Research Institute of Texas (RP110595 and RP120214), the Ovarian Cancer Research Fund Program Development Grant, Department of Defense grants (OC120547 and OC093416), the Betty Ann Asche Murray Distinguished Professorship, the RKG Foundation, the Gilder Foundation, the Judd A. Rees Ovarian Cancer Research Fund, the Chapman Foundation, the Meyer and Ida Gordon Foundation (to A.K. Sood), Cancer Research UK (to A.L. Harris), and the Ann Rife Cox Chair in Gynecology (to R.L. Coleman), and the Blanton-Davis Ovarian Cancer Research Program (to A.K. Sood and R.L. Coleman). R.A. Previs was supported by NIH T32 Training Grant CA101642.

Received August 21, 2014; revised September 23, 2014; accepted October 1, 2014; published OnlineFirst November 11, 2014.

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www.aacrjournals.org
Clin Cancer Res; 21(5) March 1, 2015
OF5


Clinical Cancer Research

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Clin Cancer Res  Published OnlineFirst November 11, 2014.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-0809

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