Molecular Pathways: Context-Dependent Approaches to Notch Targeting as Cancer Therapy

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Abstract
Recent high-throughput genomic sequencing studies of solid tumors, including head and neck squamous cell carcinoma (SCC), ovarian cancer, lung adenocarcinoma, glioblastoma, breast cancer, and lung SCC, have highlighted DNA mutation as a mechanism for aberrant Notch signaling. A primary challenge of targeting Notch for treatment of solid malignancies is determining whether Notch signaling is cancer promoting or tumor suppressing for a specific cancer. We compiled reported Notch receptor and ligand missense and nonsense mutations to glean insights into aberrant Notch signaling. Frequencies of coding mutations differed for the 4 NOTCH genes. A total of 4.7% of tumors harbored NOTCH1 missense or nonsense mutations. NOTCH2, and NOTCH3 had similar overall mutation rates of 1.5% and 1.3%, respectively, whereas NOTCH4 mutations were rarer. Notch ligand genes were rarely mutated. The combined mutation frequency and position spectra of the 4 Notch paralogs across the different cancers provide an opportunity to begin to illuminate the different contributions of each Notch paralog to each tumor type and to identify opportunities for therapeutic targeting. Notch signaling pathway activators and inhibitors are currently in early clinical development for treatment of solid malignancies. Defining the status and consequences of altered Notch signaling will be important for selection of appropriate treatment.

Background
The tumor microenvironment for solid malignancies involves a complex interplay of tumor cells, stromal matrix and support cells, blood vessel endothelial cells, and immune cells. In order for solid tumors to progress and grow, an adequate blood supply is needed. The interplay between tumor cells and the endothelial cells of blood vessels will be vital to ensure the tumor is adequately supplied with nutrients. The stromal cells and matrix, originally thought to provide a relatively inert support for the process of tumorigenesis and tumor progression, have more recently been appreciated to be co-opted, active participants in these pathologic processes. Notch signaling occurs at the interface of these microenvironment compartments (Fig. 1).

There are 4 Notch family receptors in humans, Notch 1 to Notch 4. Each of the 4 Notch receptors is initially produced as a single polypeptide that is cleaved by a furin-like convertase at site 1 (S1) while in transit through the Golgi apparatus to create noncovalently attached heterodimers.

The extracellular amino-terminal portion of the Notch receptor contains a series of 29 to 36 EGF-like domains, specific subsets of which are involved in interactions with Notch ligands. A heterodimerization domain tethers the Notch extracellular domain to the carboxyl-terminal portion of the Notch receptor, which comprises an extracellular heterodimerization domain, a transmembrane domain, and Notch intracellular domain (NICD). The canonical Notch ligands include Delta-like ligand (DLL) 1, 3, and 4 and Jagged1 (Jag1) and Jagged2 (Jag2). These ligands, similar to the Notch receptors, are single-pass transmembrane proteins with numerous extracellular EGF repeats.

Notch receptors are activated by a series of proteolytic events following productive ligand binding. Several excellent recent reviews provide detailed mechanisms of Notch, including activation by noncanonical ligands (1, 2). Here, we highlight Notch domains and canonical Notch signaling pathway components currently recognized as most relevant for tumorigenesis (Fig. 1). Ligand binding can result in Notch activation when the bound ligand is expressed on a cell adjacent to the Notch-expressing cell (trans interactions) or Notch inhibition when the bound ligand and Notch receptor are expressed on the same cell (cis interactions; ref. 1). Distinct Notch EGF domains mediate the Notch-activating trans interactions and the inhibiting cis interactions with ligands (3). In addition to the EGF repeats within the extracellular domain, 3 Lin-12/Notch repeats (LNR) participate in activating cleavage at site 2 (S2), which is approximately 12 amino acids beyond the transmembrane domain, until...
it is appropriately exposed following ligand binding. Trans interactions between ligand and Notch provide the TACE/ADAM (a disintegrin and metalloproteinase) metalloproteinases access to the Notch S2, permitting cleavage at S2 and the removal of the ectodomain (1). The loss of the ectodomain results in a membrane-tethered intermediate that is a substrate for γ-secretase, a multicomponent intramembrane protease (1).

Cleavage of Notch receptors by γ-secretase is required for the release from the membrane of the NICD, which then translocates to the nucleus. The NICD regulates transcription of target genes through interactions with...
transcriptional machinery partners, including the CBF-1, Su(H), Lag-1 (CSL) DNA-binding transcription factor via the Notch ankyrin repeats and the Mastermind transcriptional coactivators (MAML1-3) via the Notch RAM motif (4). Notch 1 and Notch 2 each contain a transcriptional activation domain (TAD) following the ankyrin repeats within the NICD, but the TAD is lacking for Notch 3 and Notch 4. Located near the carboxy-terminus of the NICD is the PEST domain, which can be ubiquitinated by the FBXW7-containing E3 ubiquitin ligase complex targeting the NICD for destruction. The specific unique biologic functions of the 4 different Notch receptors and how these activities differ by ligand are not well understood.

Signaling events in Notch ligand–expressing cells are likely important, though as yet poorly understood contributors to tumorigenesis. Similar to Notch receptors, Notch ligands undergo sequential proteolytic cleavage following receptor binding and have been reported to activate transcription (5). An intact PDZ- (PSD-95/DLG/ZO-1)-ligand domain, which is present at the carboxyl-terminus of Notch ligands, was reported to be essential for Jag1-conferred transformation of immortalized rat kidney cells in vitro (6). The DLL4 Notch ligand is highly expressed at the tips and stalks of sprouting vessel endothelial cells and is an important regulator of angiogenesis (7).

The Notch signaling pathway has long been appreciated as functioning in developmental processes and regulating the self-renewal of tissues. More recently, the role of Notch signaling in cancers has been reported, with Notch signaling having both oncogenic and tumor-suppressive roles, depending on the cellular context. Activating mutations in NOTCH1 were identified in T-cell acute lymphoblastic leukemia and chronic lymphoblastic leukemia (8–10), implicating NOTCH1 as an oncogene for these hematopoietic cancers. In the absence of reported NOTCH mutations in solid malignancies before 2011, there was already an appreciation that the contribution of Notch signaling to tumor development was largely context dependent, with Notch contributing to tumor development in some instances, whereas suppressing tumorigenesis in other contexts. There have been a number of excellent recent reviews discussing these nuances of Notch signaling (11–14). Our goal for this article is to highlight available NOTCH mutation and published functional/correlative data for solid malignancies to begin to more fully appreciate the different roles of Notch signaling in cancer and opportunities for therapeutic targeting.

**NOTCH receptor mutations in solid malignancies**

A number of groups have investigated Notch receptor and ligand mutation status in various tumor types either through whole-exome sequence analysis [head and neck squamous cell carcinoma (HNSCC; refs. 15, 16), ovarian carcinoma (17), and colorectal and breast cancers (18)], or by sequence analysis of entire candidate gene exons, including Notch receptors and ligands (breast cancer; ref. 19), glioblastoma (20), lung adenocarcinoma (21), and lung squamous cell carcinoma (lung SCC; ref. 22). Review of these data for nonsilent protein–coding mutations indicated that Notch ligands were rarely mutated; JAG1 was mutated in 1 of 91 sequenced glioblastomas, and JAG2 was mutated in 2 of 188 lung adenocarcinomas (20, 21). Notch receptors were mutated in several tumor types, and the frequency of mutation of specific Notch receptors and the location of the mutations within each Notch receptor varied markedly by tumor type (Fig. 2). We confined our analysis to nonsilent protein coding alterations whose protein product could be predicted and did not evaluate splice site mutations. Mutations that were reported to be tested but were not confirmed in validation studies were excluded.

Specific functional consequences of mutated Notch receptors and ligands are largely not yet experimentally defined, but the clustering of mutations in known functional elements invites speculation with regard to whether the specific Notch receptor likely functions as a tumor suppressor or oncogene in a specific cancer type (Fig. 2).

More NOTCH1 gene mutations were observed than mutations in the other NOTCH receptor genes. This was in part, but not entirely, due to the greater number of tumors with NOTCH1 sequencing data. For HNSCC, lung SCC, and breast, NOTCH1 mutations were relatively frequent, with 5% to 15% of tumors harboring protein coding changes. Many of these missense mutations occurred at or near identified important domains such as the ligand-binding domain (EGF repeats 11 and 12) or the ankyrin domains (Fig. 2). Nonsense mutations observed in HNSCC would be predicted to result in truncated Notch1 proteins lacking domains important for transcription activation. The data suggest that Notch1 may be acting as a tumor suppressor in these tumor types. Although it is formally possible that secreted truncated Notch1 proteins may be gain of function mutants, there are no data to support this notion.

Despite the prevalence of NOTCH1 mutations in HNSCC, the published data are sparse and conflicting with regard to the function of Notch1 in these tumors. Notch1 protein levels have been found to be increased in HNSCC compared with adjacent mucosal tissues (23), and HNSCC tumors expressing higher levels of Notch1 protein were associated with reduced patient survival (24). However, in separate studies, the expression of an activated form of Notch1, the NICD, was reported to result in enhanced or greatly diminished HNSCC cell line in vivo tumorigenicity (24, 25).

The increased frequency of NOTCH1 mutations in lung SCC compared with lung adenocarcinoma and the close proximity of 2 of the 3 lung SCC mutations to the ligand-binding domain invite speculation that Notch1 is more likely to function as a tumor suppressor in lung SCC than adenocarcinoma. These lung SCC mutations had a distribution that was similar to cutaneous SCC mutations identified in the same published study, a subset of which was characterized to be loss-of-function mutations by a
cell-based transcription reporter assay (22). Possible differences in Notch1 function in lung adenocarcinoma versus lung SCC are highlighted by a single report that high tumor Notch1 protein levels were associated with significantly reduced survival for lung adenocarcinoma patients ($P = 0.004$, $n = 111$), but not for lung SCC patients ($P = 0.23$, $n = 188$; ref. 26). However, reported functions of Notch1 in lung adenocarcinoma are conflicting (27, 28).

Although NOTCH1 mutations in breast cancer include a missense mutation within the ligand-binding domain and a spectrum that does not differ appreciably from those observed in HNSCC, Notch1 studies in breast cancers have generally reported functional or tumor molecular data implicating Notch1 as an oncogene in these cancers (29, 30). NOTCH1 mutations occurring in glioblastoma were infrequent and did not map to known functional domains. The functional/correlative data for Notch1 in glioma from 2 independent reports consistently implicate NOTCH1 as an oncogene (31, 32). One of the observed NOTCH1 mutations in ovarian carcinoma, P2417A, has also been observed in acute T-lymphoblastic leukemia and maps within PEST domain (33).

The Notch3 ligand–binding domains are presented in Fig. 2, as recently defined by functional studies using a competitive peptide library screen to include EGF repeats 7–10 and 21–22 (34). Mutations observed in HNSCC, ovarian cancer, and lung adenocarcinoma occurred in about 1% of these tumors and generally resided within or near recognized functional domains, suggesting these mutations may be loss-of-function mutations. NOTCH3 sequence data were not yet available for lung SCC.
The implications of the spectrum of mutations for NOTCH2 and NOTCH4 receptors were more veiled. The ligand-binding domain of Notch2 has been defined on the basis of extrapolation from Notch1. No NOTCH2 mutations were observed in the putative ligand-binding domain. The Notch4 ligand–binding domain has not been defined. Nonsense mutations observed in HNSCC and lung SCC suggest that Notch2 may function as a tumor suppressor in these cancer types. The observation of mutations in FBXW7 in approximately 5% of HNSCC in 2 independent studies and 1% of ovarian cancers is a reminder that while considering mutation spectra, there exist other important aspects of Notch regulation that cannot be gleaned from these data (15–17). Of course, it is possible that the functional consequences of the observed mutations will differ from predictions. More likely, there exist subgroups within each of these tumor types with differential dependence upon gain or loss of the different Notch receptor activities.

A caveat of comparing gene mutation profiles in which the number of tumors varies appreciably by cancer site is bias due to sample selection. Relatively small sample sizes may not reflect the study population at large and therefore subsets from each study may not reflect general characteristics. Admittedly, gene mutation spectra are but one aspect of a complex story. Projects such as The Cancer Genome Atlas (TCGA; ref. 35) will be especially informative for defining the roles of the different Notch receptors in different cancers. By characterizing tumor mutations, copy number alterations, gene expression profiles, and gene methylation patterns in a large number of tumors, it is hoped that clinically relevant subtypes of organ-specific cancers will be identified through the TCGA and other efforts and eventually lead to effective therapies as well.

Clinical–Translational Advances

Notch pathway–targeting agents in clinical development for solid malignancies

The appropriate Notch pathway–directed therapeutic is anticipated to differ depending upon whether the tumor harbors a Notch alteration resulting in gain or loss of function. An approach to generally inactivate Notch signaling via inhibition of the γ-secretase is currently being evaluated as a possible anticancer strategy for tumors with acquired Notch gain of function. γ-Secretase has been implicated in Alzheimer disease as one of the enzyme complexes responsible for cleaving amyloid precursor protein into amyloid beta, a primary component of amyloid plaques. Because of these pathogenic implications, γ-secretase inhibitors (GSI) are in clinical development for several diseases, including solid malignancies (Table 1).

### Table 1. Notch pathway–targeted agents in clinical development for solid malignancies

<table>
<thead>
<tr>
<th>Pathway alteration</th>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Company</th>
<th>Phase</th>
<th>Cancer types</th>
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<tr>
<td>Notch pathway</td>
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<td>GSI</td>
<td>Merck</td>
<td>I/I-</td>
<td>Breast, pancreatic</td>
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<tr>
<td>inhibitors</td>
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<td>GSI</td>
<td>Merck</td>
<td>I</td>
<td>Pediatric CNS</td>
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<td>GSI</td>
<td>Roche</td>
<td>I/II</td>
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<tr>
<td></td>
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<td></td>
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<td>MedImmune LLC</td>
<td>I</td>
<td>Adv. solid tumors</td>
</tr>
<tr>
<td>Notch pathway</td>
<td>LBH589; panobinostat</td>
<td>Histone deacetylase inhibitor</td>
<td>Novartis</td>
<td>II</td>
<td>Thyroid, neuroendocrine</td>
</tr>
<tr>
<td>activators</td>
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<td>NA</td>
<td>I</td>
<td>Breast, NSCLC, brain and CNS, sarcoma, NPC, adv. solid tumors</td>
</tr>
</tbody>
</table>

Abbreviations: Adv., advanced; CNS, central nervous system; NA, not applicable; NPC, nasopharyngeal cancer; NSCLC, non–small cell lung cancer, SCLC, small-cell lung cancer.

NOTE: Clinical trials were compiled from ClinicalTrials.gov.
The finding that a GSI resulted in increased incidence of skin cancers with no improvement in Alzheimer disease symptoms led to the halt of a large phase III clinical trial (36). This skin toxicity has been largely attributed to Notch inhibition, and as a result, Notch-sparing GSIs are being developed for treatment of Alzheimer disease (37–39). The accumulating preclinical and clinical data indicating that Notch is oncogenic in several tumor types has led to the recent clinical development of GSIs that are not Notch sparing for treatment of solid tumors.

Several GSIs are currently in phase I or phase I/II trials for various malignancies (Table 1). Reports from these early-phase trials indicate that biomarkers including tumor levels of Notch proteins, NICD, Notch transcriptional target genes such as HES1 and HEY1, and blood biomarker evaluations were planned for several of these studies (40–42). In general, toxicities were manageable. An intermittent dosing schedule seemed to ameliorate gastrointestinal toxicities compared with a continuous dosing regimen (40, 43). The majority of tumors were positive for Notch1 in trials reporting results (40, 41). Reported response rates were modest (40, 41) and low baseline levels of interleukin-6 (IL-6) and IL-8 were associated with clinical benefit (42).

The finding that the Notch ligand DLL4 is an important regulator of angiogenesis led to the characterization and development of DLL4-directed therapeutics as a means for interfering with tumor vascularization (Table 1). No results have been reported for the anti-DLL4 antibody therapeutic, MEDI0639. However, the finding that chronic treatment with anti-DLL4 resulted in vascular neoplasms in mice indicates that treatment management will require extra care (44).

Now that Notch1 has been identified as a tumor suppressor, at least in some tissues, administering systemic therapeutics that inhibit general Notch signaling is anticipated to require careful management to reduce additional cancer risks. This would be especially true for patients with early-stage disease in which the risks of a secondary cancer may outweigh the possible benefits.

**Notch-activating agents in clinical development for solid malignancies**

Epigenetic silencing of Notch and other tumor-suppressor genes is a prominent mechanism for tumor development. Application of agents that interfere with epigenetic silencing of NOTCH1 has been reported to reduce tumor-like phenotypes in several preclinical models, including neuroendocrine tumors, medullary thyroid cancers, and others (45–48). Histone deacetylase (HDAC) inhibitors have had some success with NOTCH reexpression in cancers in which NOTCH expression was lost by this mechanism. There are 2 HDAC inhibitors currently in clinical development (Table 1), and efforts to identify Notch pathway compounds are ongoing (48). Results from a phase II study of valproic acid (VPA) involving 8 patients with neuroendocrine tumors indicated that NOTCH1 expression was absent from tumors before treatment and increased significantly following VPA treatment. Four of these patients had stable disease as best response (49).

Tumors harboring more than one predicted NOTCH inactivating mutation are infrequent (15, 16). Formal possibilities are that the wild-type allele is present and expressed appropriately in tumors with mutated NOTCH and, therefore, loss of function NOTCH mutations would be described as haploinsufficient. Alternatively, the mutations predicted to be loss-of-function mutations are in fact gain-of-function mutations acting through an as yet unknown mechanism. An HDAC inhibitor would not be a rational solution in either case. However, if the wild-type allele is present but transcriptionally repressed, as has been observed for BRCA1 mutations in breast cancers, an HDAC inhibitor may be a viable therapeutic for tumors harboring a NOTCH-inactivating mutation. As we learn more about the context and functional consequences of specific NOTCH mutations, we will be better poised to identify appropriate therapeutics for the increasing number of solid tumors with identified NOTCH mutations.

A better understanding of the specific Notch signaling pathway alterations in the context of each cancer is warranted. We posit that the nuances and ambiguities of the roles of Notch receptor signaling in cancers may be defined, at least in part, by the specific Notch pathway mutation, Notch receptor and ligand expression alteration, and concomitant gene expression profile. Ongoing efforts such as those by the TCGA will greatly facilitate the development of the contextual understanding of Notch and other tumor-specific alterations.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** A.M. Egloff

**Writing, review, and/or revision of the manuscript:** A.M. Egloff, J.R. Grandis

Received April 20, 2012; revised June 11, 2012; accepted June 13, 2012; published OnlineFirst July 6, 2012.


