

The Role of *Notch3* in Cancer

ZVIADI ABURJANIA, SAMUEL JANG, JASON WHITT, RENATA JASKULA-STZUL, HERBERT CHEN, J. BART ROSE

Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama, USA

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ABSTRACT

The *Notch* family is a highly conserved gene group that regulates cell-cell interaction, embryogenesis, and tissue commitment. This review article focuses on the third *Notch* family subtype, Notch3. Regulation via Notch3 signaling was first implicated in vasculogenesis. However, more recent findings suggest that Notch3 signaling may play an important role in oncogenesis, tumor maintenance, and resistance to chemotherapy. Its role is mainly oncogenic, although in some cancers it

appears to be tumor suppressive. Despite the wealth of published literature, it remains relatively underexplored and requires further research to shed more light on its role in cancer development, determine its tissue-specific function, and elaborate novel treatment strategies. Herein we summarize the role of Notch3 in cancer, possible mechanisms of its action, and current cancer treatment strategies targeting Notch3 signaling. *The Oncologist* 2018;23:900–911

Implications for Practice: The *Notch* family is a highly conserved gene group that regulates cell-cell interaction, embryogenesis, and tissue commitment. This review summarizes the existing data on the third subtype of the *Notch* family, Notch3. The role of Notch3 in different types of cancers is discussed, as well as implications of its modification and new strategies to affect Notch3 signaling activity.

INTRODUCTION

The *Notch* family was discovered one century ago; however, various functions of the gene products have not been elucidated until the last few decades. In mammals, there are four Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five canonical ligands [1]. Three Delta-like ligands (DLL1, DLL3, and DLL4) are homologues in the *Drosophila* Delta family, and two Jagged ligands (Jag1 and Jag2) are homologues in the *Drosophila* Serrate family [2]. The ligand proteins on a neighboring cell's surface bind to the extracellular domain of Notch and via in-trans regulation induce a signaling cascade to control cell differentiation, proliferation, and many other processes.

All Notch proteins share a similar basic structure, including an extracellular domain (ECD) at the N-terminus, a transmembrane domain (TMD), and the Notch intracellular domain (NICD) at the C-terminus. The ECD of Notch includes 29–36 epidermal growth factor (EGF)-like repeats that contain six residues of cysteine, which favor the formation of a stable loop-like structure. The Notch ligands bind these EGF-like repeats with high specificity. The TMD contains a conserved negative regulatory region, which consists of three Lin-Notch repeats that form a complex with Fringe, a protein modulating the ligand preference of Notch, and one transmembrane region [3]. The NICDs of all Notch family proteins contain three conserved domains: C-repeat/DRE binding factor 1 (CBF1)/Recombination Signal Binding Protein For Immunoglobulin Kappa J Region (RBP-jk)-associated molecule (RAM), seven Ankyrin repeats (ANK), and

proline-glutamic acid-serine-threonine (PEST). Most proximal to the cell membrane is the RAM domain, which interacts with CBF1/Suppressor of Hairless/gene/protein required for Lin-12 and glp-1 signaling (Lag-1) (CSL) to form a transcription complex. Both the ANK domain and the PEST sequence are located near the C-terminus and are involved in regulating protein stability and degradation. Only Notch1 and Notch2 include a transactivation domain (TAD) between ANK and PEST [4].

The Notch pathway leads to the expression of its target genes via a series of translocation, post-translational modifications, and activation by its ligands. After translation, the Notch receptor is modified in the Golgi via proteolytic cleavage at site 1 (S1) by Furin-like convertase. It is then transported to the cell surface as a heterodimer held together by noncovalent interactions. The Notch receptor on the signal-receiving cell is activated by binding to a ligand on the cell surface of a neighboring signal-sending cell. The binding causes a conformational change of the receptor to expose site 2 (S2) for cleavage by a disintegrin and metalloprotease (ADAM). Notch cleavage at S2 generates the membrane-anchored Notch extracellular truncation (NEXT) fragment, a substrate for the γ -secretase complex. γ -secretase then cleaves the Notch transmembrane domain in the NEXT fragment progressively from site 3 (S3) to site 4 (S4), thereby releasing the NICD into the cytoplasm. NICD then enters the cell nucleus, where it associates with the DNA-binding protein CSL. In the absence of NICD, CSL may associate with ubiquitous

Correspondence: J. Bart Rose, M.D., 1510 20th Street South, Birmingham, Alabama 35294-3411, USA. Telephone: 205-975-3984; e-mail: jbrose@uabmc.edu Received December 22, 2017; accepted for publication February 19, 2018; published Online First on April 6, 2018. <http://dx.doi.org/10.1634/theoncologist.2017-0677>

corepressor proteins and histone deacetylases (HDACs) to repress transcription of the target genes. However, in the event of NICD binding, allosteric change occurs in CSL that facilitates the displacement of transcriptional repressors. Subsequently, transcriptional coactivator Mastermind (MAM) recognizes the NICD/CSL interface, and the complex recruits additional coactivators mastermind-like protein (MAML) and p300/P300/CBF-associated factor (PCAF) to activate transcription. The aforementioned Notch pathway is depicted in Figure 1.

Notch signaling plays an important role in cell fate determination and organogenesis. Although the focus of this review is on Notch3, each of the other subtypes has been linked to human diseases. Missense mutations in the *Notch1* gene causes bicuspid aortic valve formation, and its deletion is lethal during early stages of embryogenesis [5, 6]. It has also been shown to have both oncogenic and tumor suppressor roles in different types of cancers [7, 8]. Mutations in the *Notch2* gene can cause Alagille and Hajdu-Cheney syndromes, two autosomal-dominant genetic conditions that affect skeletal, cardiovascular, neural, and gastrointestinal system development [9, 10]. An association between the mutation of the *Notch4* gene and susceptibility to schizophrenia has been described [11].

The Notch3 gene, identified as the third mammalian Notch, was initially described in proliferating neuroepithelium [12]. Located on chromosome 19p13.12 (19: 15159632-15200980), its 33 exons encode a single-pass transmembrane heterodimer receptor protein composed of 2,321 amino acids. In canonical Notch3 signaling, Notch3 intracellular domain (NICD3) travels to the nucleus, where it binds to CSL to replace the transcription repressors HDAC1 and silencing mediator for retinoid or thyroid-hormone receptors (SMRT) and recruits coactivators to activate transcription of *Hey* and *Hes1*, both of which are transcriptional regulators of the basic helix-loop-helix class.

A unique aspect of the Notch3 subtype is its restricted pattern of tissue distribution. Notch3 expression has been found in vascular smooth muscle, the central nervous system, and subsets of thymocytes [12–14]. Although the deletion of Notch1 and Notch2 is lethal to a developing embryo, deletion of Notch3 is not [5, 15]. Despite sharing a similar basic structure with Notch1 and 2, Notch3 displays a number of structural differences. The most apparent difference is a missing TAD, which could account for the weak transactivation activity of the NICD3 [4]. Slight differences are also evident in the Notch3 extracellular domain. It specifically lacks the Notch 1 and 2 equivalent of EGF repeat 21 and parts of EGF repeats 2 and 3 [12]. These differences could explain the differential ability of NICD3 to recruit coactivators or corepressors and undergo distinct conformational changes.

Changes in Notch activity have been associated with several benign and malignant diseases [7, 16–21]. Although the primary function of Notch seems to be regulation of vasculogenesis, in which its dysregulation has been linked to a number of vasculopathies, it can also have tumor suppressive or oncogenic roles (Table 1).

NOTCH3 AND VASCULOGENESIS

Although Notch3 is almost undetectable in normal human microvascular endothelium, it is elevated in infantile hemangioma stem cells [22]. The importance of Notch3 in development of normal vasculature was suggested when Notch3-null mice

had decreased retinal neovascularization and increased Notch3 expression was shown in a hypoxic model [23]. Kaposi's sarcoma is a virus-induced angioproliferative disease, and the protein that is necessary for virus reactivation has been shown to form a complex with RBP-J κ [24, 25]. Interestingly, the endothelial cells in lymphatic vessels infected with Kaposi's sarcoma herpesvirus had higher Notch3 receptor density compared with uninfected cells [26]. Additionally, it has been demonstrated that endothelial Jag1 can modulate Notch3 signaling in the perivascular smooth muscle layer with a proangiogenic effect [27].

CEREBRAL AUTOSOMAL-DOMINANT ARTERIOPATHY WITH SUBCORTICAL INFARCTS AND LEUKOENCEPHALOPATHY

Because of the distribution pattern of Notch3, abnormalities in this protein are linked primarily to vascular pathologies, such as small-vessel disease in skin, coronary arteries, and the brain [21]. The first example of Notch3-related disease was reported in 1993, when mutations in the extracellular domain were identified in patients with cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [28]. Since then, over 70% of mutations in CADASIL have been reported to occur in EGF-like repeats 3 and 4 of the Notch3 extracellular domain. Nearly all are missense mutations involving a cysteine residue, in which cysteine is replaced with another amino acid or vice versa. The odd number of cysteine residues are thought to lead to misfolding of the protein, ultimately affecting its trafficking, processing, ligand binding, and signal transduction. Three mechanisms that involve mutant Notch3 in the pathology of CADASIL have been explored:

- Local accumulation of Notch3 extracellular domain in the vascular smooth muscle results from local aggregation and impaired endocytosis [14]. Accumulated mutant Notch3 prevents protein ubiquitination and degradation, which leads to the formation of granular osmiophilic material [29].
- The mutations in the Notch3 extracellular domain lower its affinity to Furin and thereby reduce the frequency of S1 cleavage. The amount of the mutant Notch3 transported to the cell surface is reduced and subsequently results in intracellular aggregation [30].
- Mutations in EGF repeats 10 and 11 may alter CBF1/RBP-j κ interaction. Interestingly, there are reports that CADASIL mutant Notch3 receptors show no change in affinity for ligand binding and that some mutations may actually increase signaling [30].

NOTCH3 IN HYPOXIA AND INFLAMMATION

Chronic inflammatory conditions have been associated with development of cancer in the gastrointestinal tract [31]. The inflammatory process involves the interplay between proinflammatory cytokines (e.g., interleukin 6 [IL6]) and a variety of anti-inflammatory mediators that may be produced by enzymes such as 15-lipoxygenase-1 (15-LOX-1). The constitutive activation of 15-LOX-1 in mice intestine decreased IL6 expression and signal transducer and activator of transcription 3 (STAT3) phosphorylation. Decreased IL6/STAT3 transcriptional activation resulted in reduced Notch3 expression and concomitant inhibition of colitis-associated colorectal cancer development [32]. IL6-mediated induction of Notch3 has also been

Table 1. Oncogenic and tumor suppressive roles of Notch3

Tumor type	Observation	Species	References
Tumors in which Notch3 signaling has an oncogenic function			
Hemangioma	Expression of Notch3 is elevated in infantile hemangioma stem cells	Human	22
Kaposi's sarcoma	Endothelial cells in lymphatic vessels that were infected with Kaposi's sarcoma herpesvirus had higher Notch3 receptor density compared with uninfected cells	Human	26
T-ALL	Activation of Notch3 signaling is associated with cancer survival and proliferation	Human	45–47
	Both Notch3 knockdown and γ -secretase inhibitors reduced MKP-1 levels, which led to impaired tumorigenicity and increased apoptosis of T-ALL	Human	48
T-cell lymphoma	Transgenic mice that express Notch3 intracellular domain develop T-cell lymphomas attributable to Notch3-induced NF- κ B activation	Mouse	50
Nasopharyngeal carcinoma	Knocking down Notch3 in nasopharyngeal carcinoma increased chemosensitivity to cisplatin	Human	56
SCC	Notch3 expression is higher in SCC compared with adjacent normal tissue	Human	55, 57
	Notch3 is associated with the aggressiveness of esophageal SCC and resistance to 5-FU	Human	58
	Injection of γ -secretase inhibitor caused cervical SCC shrinkage in mice	Mouse	57
Breast cancer	Notch3 amplifications are more common than mutations in TNBC	Human	65
	In TNBC, mutations have been seen in PEST domain, which is involved in the negative regulation of gene expression	Human	64
	Notch3 causes mammary tumor development in transgenic mice; inhibiting Notch3 in vitro with γ -secretase inhibitors or with Notch3 siRNA was shown to induce apoptosis in inflammatory breast cancer	Mouse	61
	Notch3 is associated with higher invasiveness and metastatic rate in breast cancer	Human	8, 35, 36, 70, 71
	Notch3 is associated with chemoresistance	Human	72
Ovarian cancer	Notch3 expression is significantly higher in ovarian cancer compared with normal ovaries or benign ovarian tumors	Human	17, 78
	Notch3 expression was associated with higher tumor grade, lymph node and distant metastasis, and higher clinical stage	Human	17, 78, 82
	High Notch3 expression is associated with tumor recurrence and poor survival	Human	17, 78
	High Notch3 expression is associated with chemoresistance	Human	80, 83–86
Colorectal cancer	Notch3 is commonly expressed at a significantly higher level in colorectal cancer compared with normal tissue	Human	92, 93
	Decreased IL6/STAT3 transcriptional activation resulted in reduced Notch3 expression and concomitant inhibition of colitis-associated colorectal cancer development	Human	32
	Notch3 is associated with invasiveness and spreading of the tumor	Human	94, 92, 95
	Notch3 is associated with chemoresistance and worse clinical outcomes	Human	92, 148, 149
HCC	Notch3 is elevated in HCC compared with normal adjacent tissue. High Notch3 expression is associated with larger tumors and elevated α -fetoprotein levels.	Human	97, 98, 102
	Infection with HBV is associated with high Notch3 expression in HCC	Human	99, 100
	Notch3 is associated with invasiveness and spread of HCC	Human	96, 98, 100–103
PDAC	Notch3 is associated with invasiveness and spread of PDAC	Human	107–109
	Notch3 is associated with chemoresistance of PDAC	Human	113
	Notch3 expression has been correlated with shortened disease-free and overall survival	Human	108, 112

(continued)

Table 1. (continued)

Tumor type	Observation	Species	References
Prostatic adenocarcinoma	Notch3 expression was found to be positively correlated with Gleason score, and high expression of Notch3 was seen in prostate cancers with high metastatic potential	Human	40, 41
	Notch3 is associated with chemoresistance of prostate adenocarcinoma	Human	43
NSCLC	Overexpression of Notch3 has been found in 40% of NSCLC	Human	122, 123
	Notch3 increases tumor formation in genetically engineered mouse models	Mouse	124
	Notch3 inhibited apoptosis and decreased cell proliferation	Human	122, 123, 125, 126
	Notch3 is associated with tumor invasiveness, spread, and poor clinical outcome	Human	125, 127–130
	Notch3 is associated with chemoresistance and resistance to radiotherapy	Human	38, 124, 125
Tumors in which Notch3 signaling has tumor-suppressive function			
Breast cancer	Notch3 can increase the chemosensitivity in doxorubicin-resistant breast cancer	Human	68
Ovarian cancer	Notch3 is associated with better clinical outcomes	Human	90, 91
	Induction of the Notch3 intracellular domain led to p27kip1 accumulation that resulted in cancer cell cycle arrest at the G0/G1 phase	Human	66
	Notch3 induces apoptosis and stops cell proliferation	Human	79, 80
Small cell lung cancer	The lower expression of Notch3 in SCLC compared with normal lung tissue might suggest that Notch3 has tumor suppressive role in this type of cancer	Human	138, 141, 143
NETs	Notch3 has antiproliferative and proapoptotic effect on NETs	Human	117–120
	Notch3 decreases neuroendocrine tumor markers	Human	115, 117

Abbreviations: 5-FU, fluorouracil; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IL6, interleukin 6; MKP-1, mitogen-activated protein kinase phosphatase 1; NET, neuroendocrine tumor; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NSCLC, non-small cell lung cancer; p27kip1, PDAC, pancreatic ductal adenocarcinoma; PEST, proline-glutamic acid-serine-threonine; T-ALL, T-cell acute lymphoblastic leukemia; SCC, squamous cell cancer; siRNA, small interfering ribonucleic acid; STAT3, signal transducer and activator of transcription 3; T-ALL, T-cell acute lymphoblastic leukemia; TNBC, triple-negative breast cancer.

reported to promote stem cell-like features, spheroid formation, and fluorouracil (5-FU) resistance [33].

The association between IL6 and Notch3 has been observed in many types of tumors other than those of the gastrointestinal tract. Epidermal growth factor receptor (EGFR)-enriched basal breast tumors have been found to have high IL6 and Notch3 expression [34]. IL6 increases Notch3 through the microtubule associated protein kinase (MAPK) pathway that leads to mammosphere formation and increase of Carbonic anhydrase 9 (CA-IX), an enzyme involved in survival during hypoxia [35]. Notch3 is activated during hypoxia in prostate cancer cells, and Notch3-Hypoxia-inducible factor 1- α (HIF-1 α) interaction is necessary for induction of CA-IX in breast carcinoma cells [36, 37]. Notch3-HIF-1 α interaction has also been found to be important in hypoxia-related radiation resistance in lung cancer [38].

UROGENITAL CANCERS

One of the tumors that has been found to be associated with CADASIL syndrome is prostatic adenocarcinoma [39]. Prostate cancer has been reported to have upregulated Notch3 and its downstream target gene *Hey1* [20]. Notch3 expression was found to be positively correlated with the Gleason score, and high expression of Notch3 was seen in prostate cancers with high metastatic potential [40, 41]. In addition, endothelial Jag1

can activate Notch3 signaling in prostate tumor, leading to cancer cell proliferation and dedifferentiation [42].

Notch3 was shown to contribute to drug resistance when Notch3 knockdown combined with cisplatin chemotherapy eradicated drug-resistant urothelial cancer cell clones. Notch3 knockdown also reduced bladder cancer progression in vivo, an observation perhaps most suggestive that Notch3 could be a suitable chemotherapeutic target for treating urothelial cancer [43].

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LEUKEMIA

Notch3 is expressed in the early stages of T-cell development, but it normally becomes downregulated as cells transition to the double-positive stage during maturation. Furthermore, Notch3 appears to be redundant for T-cell development, whereas Notch1 is required [44]. Even so, Notch3 expression is found in the majority of T-cell acute lymphoblastic leukemia

(T-ALL) cases. Although it is rarely mutated, Notch3 appears to play a significant role in the survival and proliferation of T-ALL [45]. For example, when expressed in endothelial cells, DLL4, another Notch activator, promotes T-ALL cell proliferation by activating Notch3 signaling [46].

Recently, it has been shown that prostate apoptosis response factor 4 and Thanatos-associated domain-containing apoptosis-associated protein 1 cooperate to induce apoptosis in T-ALL cells and that this is mediated through the increased expression of cell cycle and apoptosis regulator 1 (CCAR1). However, Notch3 binding to the promoter region of CCAR1 leads to a truncated form of the protein and attenuates the proapoptotic function of CCAR1 [47]. Increased Notch3 signaling also plays a role in both the aggressiveness and the survival of T-ALL through the Ras/MAPK pathway. Although it does not appear to be a direct transcriptional target, the protein stability of mitogen-activated protein kinase phosphatase 1 (MKP-1) appears to be under the control of Notch3. Both Notch3 knockdown and γ -secretase inhibitors reduced MKP-1 levels, which led to impaired tumorigenicity and increased apoptosis [48].

Next to Ras/MAPK pathway, NF- κ B is perhaps one of the most commonly reported transcription factors interacting with the Notch signaling pathway. It has been shown that transgenic mice that express the Notch3 intracellular domain develop T-cell lymphomas attributable to Notch3-induced NF- κ B activation and that protein kinase C θ was a downstream target of Notch3 responsible for NF- κ B activation in T-cell leukemia [49, 50].

As stated previously, Notch signaling can have either oncogenic or tumor suppressor roles. The silencing of tumor suppressor genes has been linked to the overexpression of some HDACs [51]. HDAC1 is involved not only in histone modifications but also as part of a corepressor complex in the Notch signaling [52]. However, both HDAC1 and p300 also directly interact with Notch3 via modulation of acetylation. The transcription factor p300 contains an acetyltransferase domain that promotes ubiquitination and subsequent degradation of Notch3, whereas HDAC1 can reverse this by deacetylation. Agents that inhibit HDAC favored p300 activity leading to hyperacetylation. T-cell proliferation was enhanced in cells possessing a nonacetylatable Notch3 mutant and was inhibited in cells possessing acetylatable Notch3 after treatment with HDAC inhibitors (HDACi) [53]. Immunoprecipitation experiments showed that HDAC1 directly interacts with Notch3. In another study, HDACi did not change the levels of Notch1 protein or downstream gene expression in B-cell acute lymphoblastic leukemia cells. However, cell proliferation was inhibited, cell cycle was blocked in the G1/S phase, and apoptosis was induced [54]. These results suggest that although Notch1 may be important in the initiation of leukemia, more work needs to be done to more fully appreciate the role Notch3 has in leukemic cell survival and disease progression.

SQUAMOUS CELL CARCINOMA

Squamous cell carcinoma (SCC) has higher Notch3 expression compared with surrounding normal epithelial tissues. Examination of 74 pathologic specimens found that Notch3 expression was higher in SCC compared with adjacent normal tissue [55]. Notch3 overexpression was seen in 18.2% of head and neck squamous cell cancers, in more than 92% of primary nasopharyngeal carcinomas, and in all Epstein-Barr virus (EBV)-positive tumor

cell lines [56]. Cervical SCC had higher nuclear Notch3 immunoreactivity compared with normal cervical epithelium or cervical intraepithelial neoplasms [57]. Additionally, increased Notch3 mRNA expression was found in esophageal SCC cell lines compared with noncancerous squamous esophageal cell lines. This increased expression was associated with esophageal cancer cell aggressiveness and resistance to 5-FU [58]. Knocking down Notch3 in nasopharyngeal carcinoma increased chemosensitivity to cisplatin [56].

Higher Notch3 expression was observed not only in SCC cells but also in cancer-associated fibroblasts. Notch3 expression in normal human dermal fibroblasts was induced by direct cell-cell contact with oral SCC cell lines. Additionally, microvessel density in oral SCC was associated with the expression of Notch3 in cancer-associated fibroblasts [59].

The majority of published results suggest that high expression of Notch3 is associated with a poorer prognosis in SCC [60]. Patients with cervical SCC had a shorter overall survival if they had nuclear Notch3 expression compared with those who did not. Knocking down Notch3 had antiproliferative and apoptotic effect on cervical SCC, and the injection of γ -secretase inhibitor in tumors caused tumor shrinkage in mice [57]. An increase of Notch3 expression in one third of oral SCC has been correlated with larger tumor size. Growth inhibition and induction of apoptosis were observed in nasopharyngeal carcinoma after Notch3 knockdown and was associated with a decrease in protein levels of CCND1, C-MYC, NFKB1, BCL2, BCL-XL, and SURVIVIN.

BREAST CANCER

In breast cancer, Notch3 serves mainly as an oncogene with some exceptions. Notch3 causes mammary tumor development in transgenic mice [61]. In breast cancer cell lines, Notch3 signaling was constitutively active, and among other Notch receptors its activity was solely sufficient for promoting tumor growth both in vitro and in vivo [16]. The importance of Notch3 signaling among other Notch family members was also shown by a study that demonstrated that downregulation of Notch3 significantly suppressed proliferation and promoted apoptosis of the ErbB2-negative tumor cell lines. This effect was not observed in the ErbB2-positive tumor cells. In contrast, Notch1 siRNA did not suppress the proliferation of either ErbB2-positive or ErbB2-negative cell lines [62]. Notch3 activation was seen in a human inflammatory breast cancer xenograft model by RT-quantitative reverse transcription polymerase chain reaction (qPCR), western blot, and immunohistochemistry [63]. Among Notch3 aberrations, amplifications are more common than mutations in triple-negative breast cancer (TNBC), with mutations mostly involving the PEST domain (typically involved in the negative regulation of gene expression) [64, 65]. In TNBC samples, 34% of the tumors were positive for NICD3 compared with 4% positive for Notch1 intracellular domain (NICD1) [16]. However, the role of Notch3 in breast cancer is not fully resolved. One study suggested that induction of the Notch3 intracellular domain led to p27kip1 accumulation, resulting in cancer cell cycle arrest at the G0/G1 phase [66].

The role of Notch3 in epidermal-to-mesenchymal transition (EMT) of breast cancers is also controversial. While some investigators reported that Notch3 promotes tumor aggressiveness by inducing EMT, other investigators demonstrated that Notch3 actually opposes it. Notch3 was shown to be inhibiting EMT by

upregulating the Hippo/Yap pathway [16, 67]. Another mechanism of EMT inhibition was reported to be the downregulation of Fos-related antigen 1 (Fra1), which is an activator of EMT. This observation was made in Adriamycin-resistant human breast cancer cells, in which Notch3 was found to be downregulated, whereas Fra1 was expressed at high levels [68]. On the other hand, in metastatic breast cancer cells, a decrease in Notch3 levels decreased levels of the EMT-associated proteins Snail, fibronectin, and vimentin [69].

Notch3 appears to be promoting breast cancer metastasis. It is expressed at significantly higher levels in invasive cancer compared with ductal carcinoma in situ [70]. Truncated-Fms Related Tyrosine Kinase 1 (FLT1) isoform associated with increased breast cancer invasiveness was upregulated by Notch1 and Notch3 [8]. Lymphovascular emboli of human inflammatory breast cancer have been found to express increased levels of Notch3. After transfection with NICD3, spheroid formation was detected in normal breast epithelial cells that was not seen after NICD1 transfection [71]. Selective increase of Notch3 expression in human breast cancer cell lines was seen after stimulation with transforming growth factor beta 1 (TGF β 1) secreted by bone marrow osteoblasts. Cancer cell colony formation was inhibited after anti-TGF β 1 antibody treatment and Notch3 chemical inhibition. Notch3 expression was also shown to be associated with osteolytic bone lesions [69].

Another mechanism by which Notch3 can contribute to the aggressiveness of breast cancer is its involvement in cell metabolism. Notch3 activity is essential for the survival of hormone therapy-resistant metastatic breast cancer. It was also shown to be promoting mitochondrial activity and exit from metabolic dormancy. Tamoxifen-resistant cells showed reduced mitochondrial DNA copy number, mitochondrial antigen expression, and diminished oxidative phosphorylation. The reduction of mitochondrial activity was reversed by IL6 treatment that was mediated by Notch3 [72]. The essential role of Notch3 in reversal of tamoxifen-induced mitochondrial toxicity was demonstrated when IL6 treatment failed to increase mitochondrial activity in Notch3 knockdown cells [72]. Several studies have reported that in metastatic breast cancer, functional Notch3 is essential for IL6 to maintain high levels of carbonic anhydrase, an enzyme associated with hypoxia survival and cancer invasiveness [35, 36].

Notch3 can increase the chemosensitivity in doxorubicin-resistant breast cancer by negatively regulating Fra1. Fra1 has been shown to play an important role in EMT of colorectal cancer, ovarian cancer, glioma, and breast cancer cell lines [68, 73–75]. Fra1 was highly expressed in doxorubicin-resistant breast cancer cells while Notch3 was underexpressed and this resistance was mediated by Fra1-induced EMT. Adriamycin chemoresistance could be recreated in chemosensitive cells by knocking down Notch3 signaling [68]. Stromal cells can also contribute to chemoresistance by inducing Notch3 in breast cancer cells, an effect that is reversed with Notch3 siRNA or γ -secretase inhibitor (GSI) treatment [76]. Another mechanism by which cancer cells resist treatment is tumor dormancy. Upregulation of the Notch3 pathway arrested the cell cycle in G0/G1 phase and induced Hes1, Mix1, and Δ Np63 α proteins, which promote cellular quiescence [77].

OVARIAN CANCER

Notch3 expression is significantly higher in ovarian cancer compared with normal ovaries or benign ovarian tumors [17]. More

than twofold higher expression of Notch3 was detected in 63% of serous ovarian carcinomas compared with benign ovarian tissue [78]. *Notch3* gene amplification was correlated with elevated Notch3 protein expression in high-grade serous cancers. Knocking down Notch3 induced apoptosis and stopped cell proliferation only in cells with high Notch3 expression [79]. Both Notch3 siRNA and GSI treatment decreased cell survival and antiapoptotic proteins, such as cyclin D1, cyclin D3, and the B-cell lymphoma (BCL) family, and increased antiproliferative and proapoptotic proteins, such as p21, p27, Bad, Bak, Bim, Bid, and Bax [80].

Notch3 expression was associated with higher tumor grade, lymph node (LN) and distant metastasis, and higher clinical stage in ovarian cancer [17, 78]. It is known that Notch3 promotes resistance to anoikis, a process involved in programmed cell death after detachment from anchoring extracellular matrix [81]. Jag1 is a primary Notch ligand in ovarian carcinoma as well as in peritoneal mesothelial cells. Knocking down Jag1 in mesothelial cells inhibited cell proliferation and adhesion of ovarian cancer cells, suggesting that a Jag1-Notch3 interaction promoted ovarian cancer dissemination within the peritoneal cavity [82].

High Notch3 expression is associated with tumor recurrence and poor survival [17, 78]. High Notch3 expression was associated with shorter overall survival and worse progression-free survival in recurrent high-grade serous carcinoma (HGSC) of the ovary. Furthermore, Notch3 expression was associated with recurrence of HGSC after chemotherapy. Induction of Notch3 increased the concentration that inhibits cell growth by 50% (IC50) for carboplatin in low-grade serous carcinoma cell lines, and Notch3 knockdown sensitized ovarian carcinoma to chemotherapy [83]. Tumor chemosensitization after Notch3 knockdown was also shown by cisplatin and GSI combination treatment, which led to a synergistic effect on DNA damage and G2/M cell cycle arrest [84]. Additionally, a decrease in cell cycle markers, arrest in G2/M phase, and induction of apoptosis has been observed after Notch3 knockdown in combination with either platinum-based or taxane chemotherapy [80, 85, 86]. Notch3 overexpression was associated with shorter progression-free survival in patients with advanced stage ovarian cancer who were treated with taxanes or platinum-based chemotherapeutic agents [86]. Treatment of paclitaxel-resistant ovarian cancer cells with microRNA 150 (miR-150) or overexpression of miR-136 reduced Notch3 mRNA expression, reversed chemoresistance, and decreased stemness genes [87, 88]. Another mechanism of synergy between Notch3 inhibition and chemotherapy is that it decreases angiogenesis [80, 89]. Activation of Notch3 attenuates the apoptotic effect of carboplatin by inhibiting carboplatin-induced extracellular signal-regulated kinase (ERK) phosphorylation [85]. Although several studies reported that high Notch3 expression was associated with worse overall survival time, others had contradictory findings [90]. Additionally, high Notch3 expression has been correlated with better overall survival for all patients with ovarian cancer except with those who had serous cancer [90, 91].

COLORECTAL CANCER

Notch3 is commonly expressed at a significantly higher level in colorectal cancer (CRC) compared with normal tissue, with poorly differentiated tumors having higher expression levels

than well-differentiated tumors [92, 93]. Higher expression of Notch3 is associated with increased tumor growth rate, and knocking it down slows tumor growth [93]. Notch3 expression is significantly higher in micropapillary carcinomas compared with nonmicropapillary carcinomas (69 vs. 42%), with micropapillary carcinomas having more frequent lymphovascular invasion, higher tumor stage, and LN metastasis rate [94]. Thirty-eight percent of stage II and III colorectal cancers overexpressed Notch3, which was correlated with higher tumor grades, higher rates of venous invasion and LN metastasis, and shorter recurrence-free survival [92]. Notch3 was found to upregulate the exchange factor Asef, leading to increased cell migration and tumor invasiveness [95]. miR-206, a microRNA that inhibits Notch3 translation, was found to activate apoptosis, induce cell cycle arrest, and block proliferation and migration when transfected into CRC cancer cell lines [96]. miR-1 may be another microRNA regulating Notch3 with potential for treatment utility in CRC [95].

HEPATOCELLULAR CARCINOMA

Immunohistochemical analysis of Notch3 expression in liver tissue found that 78% of hepatocellular carcinomas (HCCs) had abnormally elevated Notch3 levels, whereas it was undetectable in normal liver or tissue adjacent to HCCs with chronic hepatitis. In 95% of HCCs, Notch3 mRNA was elevated compared with matching cirrhotic tissue, and Notch3 mRNA expression was in parallel with Hes6 mRNA expression [97]. Among other Notch family members, Notch3 had the highest upregulation in HCC compared with normal liver [98]. High Notch3 expression in HCC was found to be associated with hepatitis B infection [99, 100].

Initial work suggested that Notch3 exerted its effect in HCC via the β -catenin pathway. However, it was later concluded that cell proliferation and stemness in HCC was linked to Notch3 upregulation of Nanog, a transcription factor critical for embryonic stem cell self-renewal. High expression of Notch3 is also associated with larger tumors and elevated α -fetoprotein levels [98, 101, 102]. Notch3 helps maintain low differentiation and high migratory capacity in HCC [101, 102]. Elevated Notch3 expression correlates with more satellite lesions, tumor multifocality, and metastasis [98, 101]. High expression of Notch3, but not other subtypes, is associated with increased vascular invasion by HCC [100, 101]. Transient transfection of miR-206 in HCC cells decreased Notch3, Hes1, BCL-2, and MMP-9, whereas p57 and Bax levels were upregulated. Additionally, miR-206 transfection has been shown to decrease migration in HepG2 cells via inhibition of a Notch3/matrix metalloproteinase 9 (MMP-9) pathway [96, 103]. This Notch3-linked inhibition of HCC migration through decrease of MMP-9 and MMP-2 seems to be mediated by the ERK1/2 pathway [101].

Notch3 expression is associated with early recurrence and poor overall survival in HCC and was shown to oppose the antitumor effect of chemotherapy. Downregulation of Notch3 increased sensitivity to doxorubicin, cisplatin, and sorafenib [98, 99, 101, 102, 104, 105]. Inhibition of Notch3 enhanced the effect of sorafenib in vitro as well as in a mouse xenograft model and was associated with low levels of p21 [105]. A Notch3 knockdown sensitized HCC to the cytotoxic effect of doxorubicin and increased p53 mRNA, an effect not seen with Notch1 [104].

Notch3 has been found to have an oncogenic role in other liver tumors, such as cholangiocarcinoma (CCA). Tumor formation and progression was attenuated after genetic deletion of *Notch3* in CCA cell lines. In CCA, Notch3 seemed to be affecting tumor progression via a noncanonical Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K)-Akt pathway, independent of the RBPJ protein. The authors suggested that selectively targeting Notch3 via this noncanonical pathway could prevent toxicities that are known to be RBPJ dependent [106].

PANCREATIC DUCTAL ADENOCARCINOMA

Notch3 has been reported to be overexpressed in pancreatic ductal adenocarcinoma (PDAC) and is associated with higher tumor grade, lymphovascular invasion, and rates of metastasis [107–109]. Both Notch3 and Hey1 overexpression were associated with vascular invasion and increased LN metastasis [108]. Notch3 promotes invasion by modifying E-cadherin, MMP-2, MMP-9, and vascular endothelial growth factor (VEGF) expression, whereas knocking down Notch3 decreased invasiveness [107]. Notch3 also promoted angiogenesis by activating VEGF-A, MMP-9, and Angiogenin-1 [110]. Decreased Notch3 activity resulted in tumor cell apoptosis, cell cycle arrest, and the suppression of cell proliferation, invasion, and migration, each of which correlates with overexpression of miR-613. Furthermore, downregulation of miR-613 is associated with shorter overall survival, higher TNM grade, and poor differentiation of pancreatic cancer cells [111].

Notch3 expression has been correlated with shortened disease-free and overall survival in pancreatic adenocarcinoma [108, 112]. Nuclear localization of Notch3 is not normally found in pancreatic ductal tissue, but it has been observed in 44% of PDAC and 100% of unresectable tumors and has a strong correlation to pAkt expression [109]. The link between Notch3 expression and the Akt pathway in PDAC was further supported when it was shown that knocking down Notch3 decreased PI3K/Akt activity and increased pancreatic cancer cell sensitivity to gemcitabine chemotherapy [113].

NEUROENDOCRINE CANCERS

Neuroendocrine tumors (NETs) include many types of neural crest-derived tumors that secrete different biogenic amines or peptide hormones [114]. Notch signaling has been found to have a tumor suppressor role in NETs. Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor that develops from calcitonin-producing parafollicular C cells in the thyroid gland. Developing C cells express Achaete-scute homolog 1 (ASCL-1) at high levels, and the expression of ASCL-1 is lost in mature C cells [115]. ASCL-1 is a downstream target of Notch and is considered to be a neuroendocrine tumor marker [116]. Notch3 activation decreased neuroendocrine markers (chromogranin A, ASCL-1) in MTC in a dose-dependent manner. Induction of the *Notch3* gene and increased protein activity was found to be associated with apoptosis and decreased MTC cell proliferation [117]. Notch1 has been shown to have tumor suppressive role in gastrointestinal carcinoids and pheochromocytoma; however, the role of Notch3 in these tumors is understudied and requires more research [118–120].

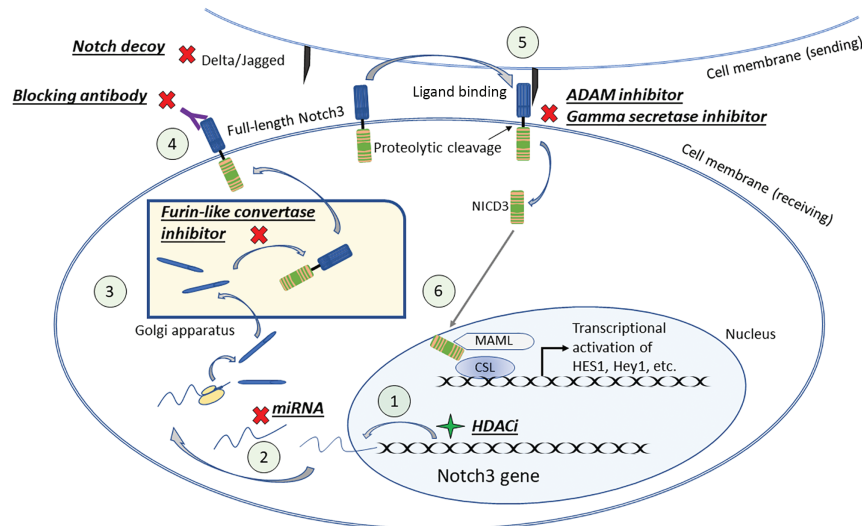


Figure 1. The Notch pathway, showing therapeutic points for targeting Notch3. (1) HDAC inhibitors can enhance Notch3 gene transcription. (2) Notch3 mRNA translation can be inhibited by miRNA. (3) Furin-like convertase inhibitors can inhibit Notch3 protein folding in the Golgi apparatus. (4) Blocking antibodies can be used to block Notch3 extracellular domain and prevent its interaction with the ligand. (5) ADAM and gamma-secretase inhibitors can be used to prevent Notch3 protein cleavage and release of Notch3 intracellular domain. (6) Notch3 is translocated to the nucleus, it binds to transcription factors and modifies target gene transcription.

Abbreviations: ADAM, disintegrin and metalloprotease; CSL, CBF1/Suppressor of Hairless/Lag-1; HDACi, histone deacetylase inhibitor; MAML, mastermind-like protein 1; miRNA, microRNA; NICD3, Notch3 intracellular domain.

LUNG CANCERS

Notch3 plays an important role in lung development. Constitutive activation of Notch3 in mice causes an increase in undifferentiated epithelial cells, delayed lung maturation, and perinatal mortality [121]. It is overexpressed in about 40% of non-small cell lung cancers (NSCLCs) [122, 123]. In genetically engineered mouse models, induced expression of Notch3 increased tumorigenicity in the lung [124]. Inhibiting Notch3 expression decreased malignant features both in vitro and in vivo via induced apoptosis and decreased cell proliferation, migration, and invasiveness [122, 123, 125]. The induction of apoptosis by Notch3 knockdown is likely mediated by Bim [126].

Clinically in NSCLC, Notch3 overexpression is associated with poorer disease-free and overall survival, higher TNM stage, and increased LN metastasis rate [125, 127, 128]. Overexpression of Notch3 induces Zinc finger E-box-binding (ZEB-1) activity, decreases E-cadherin, and upregulates fibronectin, each contributing to EMT and tumor invasiveness. This increase in EMT was abolished by ZEB-1 siRNA. Notch3 induced ZEB-1 may promote EMT and bone metastasis in NSCLC through TGF β ; however, Notch3 expression itself may be under the control of Wnt Family Member 3A (WNT3a) [129, 130].

High levels of Notch3 may cause chemoresistance and promote tumor recurrence [124, 125]. Notch3 was shown to be involved in cancer stem cell formation after lung cancer treatment with the anti-EGFR antibody erlotinib. Dramatic EGFR-mutated lung cancer cell death was seen after erlotinib treatment with consequent formation of an aldehyde dehydrogenase (ALDH)-positive stem cell-like cell population [131]. ALDH positivity has been shown to be a marker of stem-like cells that are dependent on Notch3 for tumor colony formation, and the role of notch signaling in cancer stem cell formation has been previously described [132, 133]. EGFR has been shown to decrease all subtypes of Notch proteins by protein ubiquitination [134]. Blocking EGFR led to Notch activation, which resulted in

stem cell formation. This effect was abolished by selective blockade of Notch3 but not Notch1, underscoring the importance of Notch3 in stem cell formation and suggesting that combination treatment of erlotinib with a Notch inhibitor could prevent cancer stem cell formation [124, 131]. Although not specifically targeting cancer stem cells, a combination of erlotinib with Notch3 blockade may have a synergistic effect on the induction of Bim with consequent cancer cell apoptosis [126]. Another example of successful drug combination treatment against lung cancer is pharmacologic blockade of Notch and protein kinase C α (PKC α). PKC α activates Notch3 expression, which causes asymmetric cell division leading to tumor initiation and maintenance. Treatment with gold salts selectively inhibit PKC α in combination with GSI and lead to enhanced inhibition of tumor growth compared with single drug therapy [135].

Notch3 inhibition also enhances the effectiveness of radiotherapy. Notch3 is increased during tissue hypoxia, a condition known to be a major cause of radioresistance [38, 136]. Suppression of HIF-1 α may prevent activation of the radiation-induced Notch pathway. Simultaneous inhibition of both HIF-1 α and Notch had a synergistic effect on radiation-induced lung cancer cytotoxicity [38].

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Unlike NSCLCs, small cell lung cancer (SCLC) was reported not to express Notch [137, 138]. Classical SCLC shows neuroendocrine differentiation, and Notch has been reported to be tumor suppressive in most of the neuroendocrine tumors [117, 139, 140]. The role of Notch3 signaling in SCLC is

controversial and largely unexplored. The lower expression of Notch3 in SCLC compared with normal lung tissue might suggest that Notch3 has tumor suppressive role in this type of cancer [141]. This is further supported by the finding that activation of Notch signaling in SCLC-induced cell-cycle arrest at G1 phase was associated with p21^{waf1/cip1} and p27^{kip1} induction [142]. However, these findings are somewhat contradicted by studies showing that inactivation of Notch 2 and Notch3 by monoclonal antibody decreases SCLC growth and that Notch3 expression levels do not correlate with SCLC cell proliferation [138, 143].

CONNECTIVE TISSUE TUMORS

Notch3 is upregulated in osteosarcoma and chondrosarcoma, with high expression in hypercellular areas of chondrosarcoma suggesting that it plays a role in regulating stemness in this tumor type [144]. Notch3 is overexpressed in rhabdomyosarcoma cell lines compared with normal myoblasts and has been shown to induce proliferation via a Hes1-dependent mechanism [145]. Downregulation of Notch3 in vitro resulted in increased differentiation of alveolar rhabdomyosarcoma cells and a reduction in cellular proliferation [145, 146]. It has been suggested that osteosarcoma maintains an undifferentiated state by depressing of Notch3 and its downstream target Hes5 [147].

CONCLUSION

Notch3 dysregulation has been associated with a wide variety of cancers. It has been shown to affect tumor aggressiveness, maintenance, and resistance to chemotherapy. *Notch* genes have a significant role in directing tissue commitment and cell differentiation. Activation of Notch pathways lead to increased expression of various downstream targets, some of which include transcriptional regulators. Depending on the tissue type, Notch-regulated transcriptional targets may conceivably be either oncogenes or tumor suppressors. Conflicting data within specific cancer types further suggest that the full role of Notch is not yet known. For example, it has been shown that in breast cancer, *Notch3* can act as oncogene but may also cause cell cycle arrest and prevent cellular proliferation [66, 77].

Targeted therapies can be used to modify Notch3 levels in the cells (Fig. 1). Gamma secretase inhibitors have been used to suppress Notch activity in cancers with high Notch expression, but they are not specific to Notch subtypes. Additional putative targets inhibiting Notch enzymatic processing would include

Furin-like convertase and ADAM inhibitors. To increase the specificity and avoid side effects of pan-Notch blockade, one study used monoclonal antibodies (tarextumab) to block Notch2/Notch3. MicroRNAs targeting specific Notch subtypes have been used for inhibition, including miR-136 and miR-206 for Notch3. Additionally, targeting the Notch/Jagged or Notch/Delta interaction is another route for potential therapeutic development. In cases in which Notch3 is responsible for regulating a tumor suppressor gene, it would be beneficial to induce Notch3 expression rather than inhibit it. Several histone deacetylase inhibitors have been shown to increase Notch expression in a variety of neuroendocrine tumor cells in which Notch plays a tumor suppressive role. These histone deacetylase inhibitors were shown to increase NICD3, inhibit tumor growth, induce apoptosis, and decrease tumor marker secretion. The exact mechanism by which HDACi increase Notch expression is unknown.

Modifying Notch3 activity based on Notch3 expression in tumors has been shown to have significant anticancer effect. Unfortunately, there is no clinically available laboratory test that would help us determine the level of Notch3 expression in a patient's tumor. Conceivably, immunohistochemical staining could be used on biopsy or resection specimens to determine the level of Notch3 expression and guide treatment with Notch pathway inhibitors. A less invasive method of indirectly determining Notch3 expression could be measuring serum chromogranin A (CgA) levels. CgA expression has been shown to be inhibited by the Notch pathway and has the benefit of being an established clinical test. For example, neuroendocrine tumors are known to have low Notch expression and conversely high CgA levels that decrease when treated with Notch-inducing drugs. However, this is purely theoretical, as to our knowledge, no study has correlated Notch expression to serum CgA in a clinical setting. Additional research is required to determine if this approach or others will be clinically relevant in the wide array of diseases associated with Notch3 dysregulation.

AUTHOR CONTRIBUTIONS

Conception/design: J. Bart Rose

Manuscript writing: Zviadi Aburjania, Samuel Jang, Jason Whitt, Renata Jaskula-Stzul, Herbert Chen, J. Bart Rose

Final approval of manuscript: J. Bart Rose

DISCLOSURES

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