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Tumor Suppressor Role of Notch-1 Signaling in Neuroendocrine Tumors

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Key Words. Carcinoid • Medullary thyroid cancer • Notch-1 signaling • Achaete-scute complex-like 1
Notch-1-activating compound

ABSTRACT

A growing body of literature is demonstrating that Notch signaling is a more complex process than originally thought. Contradictory findings of *notch-1* acting as an oncogene or a tumor suppressor revealed that its role is very specific to the cellular context. In this review we focus on the tumor suppressor role of Notch-1 signaling in neuroendocrine tumors (NETs) such as carcinoid and medullary thyroid cancers. NETs secrete

various bioactive hormones that can cause debilitating symptoms. Surgery is the only potential curative treatment for the patients with NETs. Notch-1 signaling is absent in these tumors and activation of Notch-1 significantly reduces tumor growth in vitro. Therefore, identification of compound(s) that activate the Notch-1 pathway in NETs could be a potential strategy to treat patients with NETs. *The Oncologist* 2007;12:535–542

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Several signaling pathways, such as the phosphatidylinositol 3' kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), and Notch-1/hairy enhancer of split 1 (HES-1)/achaete-scute complex-like 1 (ASCL-1) signaling pathway, have been shown to play important roles in regulating the growth of neuroendocrine tumors (NETs) [1–10]. Thus, a potential therapeutic target could be manipulation of these various cellular signaling pathways. One such pathway is the Notch-1 signaling pathway. Notch-1 is a multifunctional transmembrane receptor that regulates cellular differentiation, development, proliferation, and survival in a variety of contexts [11–13]. There are different but homologous Notch receptors present in diverse organisms. For example, there are four in mammals (Notch-1 to Notch-4),

two in *Caenorhabditis elegans* (Lin-12 and glucagon-like peptide 1), and one in *Drosophila melanogaster* (Notch). The differences in the structures are mainly at extracellular and cytoplasmic regions of the Notch receptors (Fig. 1). The extracellular portion has two regions with distinct functions: (a) epidermal growth factor (EGF)-like repeats, with the function of ligand binding; and (b) three cysteine-rich Notch/Lin-12 (LN) repeats, required for the blockage of signaling in the absence of ligand. The cytoplasmic extension of Notch contains a regulation of amino-acid metabolism (RAM) domain, six ankyrin repeats, two nuclear-localization signals, a transcription transactivation domain, and a proline-glutamate-serine-threonine rich (PEST) sequence. In mammals, Notch receptors vary: the extracellular regions of Notch-1 and Notch-2 contain 36 EGF repeats

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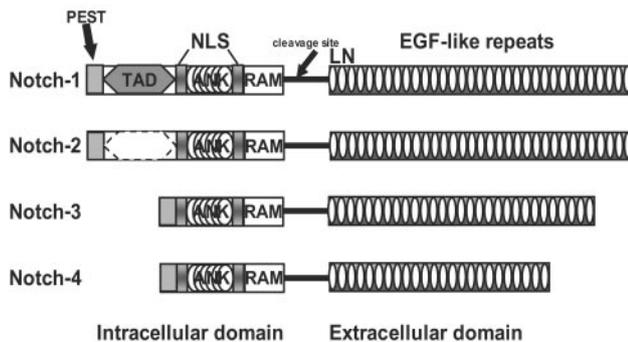


Figure 1. Organization of Notch receptors in mammals. The extracellular domain contains epidermal growth factor (EGF)-like repeats and a Lin region (LN) in all four Notch receptors. The intracellular domain contains a regulation of amino-acid metabolism (RAM), six ankyrin repeats (ANK), two nuclear localization sequences (NLS), a transcription transactivation domain (TAD), and a proline-glutamate-serine-threonine rich (PEST) sequence.

whereas Notch-3 and Notch-4 have 34 and 29 repeats, respectively. While the cytoplasmic regions of Notch-1 and Notch-2 contain a strong and weak transactivation domain, respectively, the transactivation domain is absent in Notch-3 and Notch-4 [14, 15]. Similarly, ligands that bind to the Notch receptor are also present in variable numbers. *Drosophila* has two ligands (Delta and serrate), whereas mammals have five Delta-like ligands (DLL-1, DLL-3, DLL-4, JAG-1, and JAG-2) [14, 15]. Notch-1 signaling, a highly conserved pathway throughout the animal kingdom, plays an important role in cellular differentiation, proliferation, and survival. In *Drosophila* neural development, the most studied Notch signaling pathway, Notch maintains the neural progenitor stage and inhibits differentiation [16, 17]. Recently, Notch-1 has also been shown to play an essential role in the neuroendocrine (NE) differentiation of cells in the lung and gastrointestinal (GI) tract [18–21]. Transgenic mice lacking the Notch ligand, Delta-like gene 1, or the intracellular mediator RBP-Jkappa exhibit accelerated pancreatic endocrine differentiation with a specific increase in endocrine cells in the pancreas [21]. As a result of the premature differentiation, the development of pancreas is arrested because of the reduction in precursor cells. These findings clearly demonstrate that Notch-1 signaling is required for the normal development of the pancreas [21]. Notch-1 signaling was originally thought to be a simple pathway, but recently a growing body of literature suggests that this pathway is very complex in nature. A schematic diagram of the Notch-1 signaling pathway is shown in Figure 2. Both the Notch-1 receptor and its ligands (DLL-1 and JAG-1, for example) are transmembrane proteins with large extracellular domains. Binding of any one of the Notch ligands promotes two proteolytic cleavage events in the

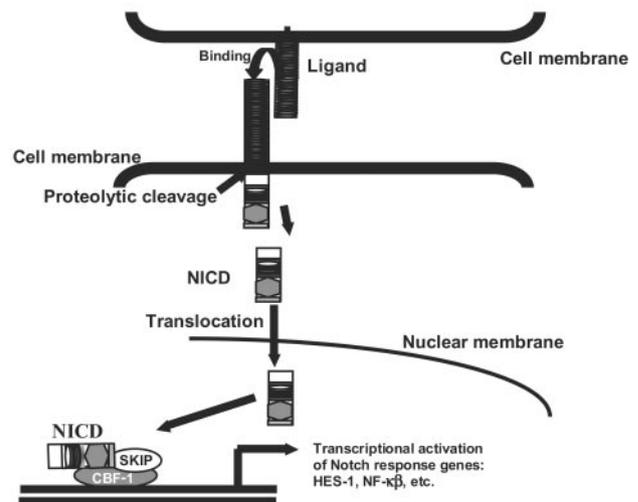


Figure 2. Schematic expression of the Notch signaling pathway. Ligand from neighboring cells binds to the extracellular region of the Notch transmembrane protein, and the simultaneous action of two proteolytic cleavages activates the intracellular domain (NICD) by releasing from the membrane. The active NICD translocates to the nucleus and binds with CBF-1 complex consisting of ski interacting proteins (SKIPs), and thus regulates various genes, including HES-1, NF- κ B, etc.

Abbreviations: CBF-1, C promoter-binding factor 1; HES-1, hairy enhancer of split 1; NF- κ B, nuclear factor kappa B.

Notch receptor resulting in the release of the Notch-1 intracellular domain (NICD) [22–24]. The first cleavage is catalyzed by a disintegrin and metalloprotease (ADAM) family proteases, whereas the second is mediated by γ -secretase, an enzyme complex that contains presenilin, nicastrin, presenilin enhancer 2, and anterior pharynx defective homologue 1 [25–27]. The released NICD then translocates to the nucleus and binds with the DNA-binding protein complex CSL (C promoter-binding factor 1 [CBF-1], suppressor of hairless, and Lag-1). The binding or the formation of the complex results in activation of various target genes such as HES-1 [23]. Activated Notch in its free form is unstable and quickly degraded, which facilitates the regulation of inappropriate activation of Notch signaling.

ROLE OF NOTCH SIGNALING IN CANCER

In human cancer cells, *notch-1* acts as either a tumor suppressor or an oncogene. The oncogenic role of *notch-1* was first identified in human T-cell neoplasia [28]. Later, it was shown that *notch-1* is upregulated in many types of cancer, including pancreatic cancer, colon cancer, non-small cell lung cancer, cervical cancer, renal cell carcinoma, and several lymphomas. These observations suggested that expression of Notch-1 signaling prevents differentiation and led to malignancy in these cancers. Furthermore, aberrant expres-

sion of Notch-1 in certain cancers inhibits apoptosis, suggesting a potential oncogenic role of *notch-1* [29–31]. However, the mechanisms by which Notch-1 inhibits apoptosis remain unclear. One possibility could be that Notch-1 cross-talks with other pathways, such as the Janus kinase, PI3K/Akt, and MAPK pathways, that might play a role in cell growth and apoptotic regulation. In addition, Notch-1 and its ligands have been shown to induce the promoter activity of nuclear factor kappa B (NF- κ B), and the downregulation of *notch-1* resulted in low levels of NF- κ B activity in keratinocytes [32]. Similarly, downregulation of *notch-1*, either by small interfering RNA (siRNA) against *notch-1* or by treatment with a small molecule compound such as genistein and curcumin, in pancreatic cancer cells resulted in growth inhibition and the induction of apoptosis [33–35]. Recently, activation of Notch-1 signaling was reported to promote the formation of human melanoma [36–38]. Conversely, Notch-1 signaling is very minimal or absent in prostate cancer, and in NETs such as small-cell lung cancer (SCLC), pancreatic carcinoid, and medullary thyroid cancer (MTC) [2, 3, 5, 14]. These apparent but paradoxical functions clearly indicate that the role of Notch signaling is dependent on its cellular context (Fig. 3 and Table 1). Therefore, the lack of Notch-1 signaling in NETs led us to examine its potential role in influencing cellular differentiation, proliferation, and/or survival in these cancers.

TUMOR SUPPRESSOR ROLE OF NOTCH-1 IN NETS

NETs such as carcinoids and MTC are difficult to treat because most patients present with advanced disease, when metastases are already present. Furthermore, patients with incurable NETs often have a poor quality of life as a result of excessive production of various bioactive hormones such as chromogranin A (CgA), serotonin, and calcitonin. In addition, NETs express high levels of ASCL-1, which seems to be limited to NETs. Disruption of the ASCL-1 transcript has been shown to affect NE differentiation, resulting in the loss of pulmonary NE cells and a decrease in NET markers [39]. Furthermore, in vivo abolition of ASCL-1 in transgenic knockout mice led to the failed development of pulmonary NE cells, a lack of thyroid C cells, a 50% reduction in adrenal chromaffin cells, and death at birth [40, 41]. These results clearly indicated that ASCL-1 is required for the development of NE cells in the body, including C cells, adrenal chromaffin cells, and pulmonary endocrine cells, the precursor cells for MTC, pheochromocytoma, and SCLC, respectively [1]. These observations in fact demonstrated that ASCL-1 is important in tumor development and it is considered to be a NET marker [1–4, 42–44]. We and others have characterized the expression of ASCL-1 in several human cancer cell lines and tumors and found that

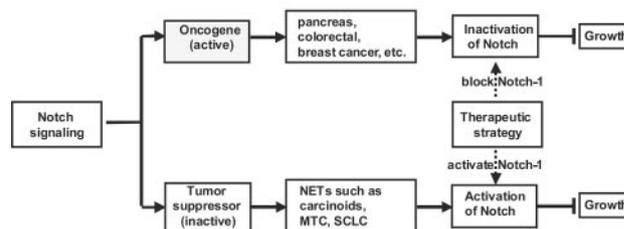


Figure 3. Summary of the Notch-1 signaling pathway in cancer. The Notch-1 role is specific to the cellular context. Abbreviations: MTC, medullary thyroid cancer; NETs, neuroendocrine tumors; SCLC, small-cell lung cancer.

ASCL-1 is, as predicted, highly expressed in NETs such as MTC, SCLC, carcinoids, and pheochromocytoma, whereas ASCL-1 is absent in non-NETs such as pancreatic cancer tissues and cell lines [1]. Furthermore, activation of the Raf-1 pathway in MTC and SCLC cells led to a significant reduction in ASCL-1 protein and growth suppression [6, 45]. Therefore, inhibition of ASCL-1 expression may be an important way to suppress NET growth. The pathways that regulate ASCL-1 expression have been well characterized. The Notch signaling pathway negatively regulates ASCL-1 during *Drosophila* and mammalian development [46]. Ligand-activated Notch-1 protein translocates to the nucleus and partners with the CBF-1 complex, and acts as a transcriptional activator for various genes, including upregulation of *HES-1*, a transcriptional repressor of ASCL-1 [23]. Interestingly, recent studies have shown that Notch-1 signaling is very minimal or nonexistent in NETs [2, 3, 5, 47, 48]. This could be the reason that we see high-level expression of ASCL-1 protein in these tumors. In this review, we focus on the role of Notch-1 signaling on growth and hormone production in carcinoids, MTC, and SCLC.

CARCINOIDS

Carcinoid tumors, the most common type of NET, are derived from enterochromaffin cells. The estimated prevalence of carcinoid tumors is one to two cases per 100,000 people, and they are most frequently found in the GI system [8]. These tumors are generally slow growing, but frequently metastasize to the liver [9]. The diagnosis of a carcinoid tumor is based on histology, with confirmation by immunohistochemical staining for NET markers such as CgA. Carcinoids secrete an excessive amount of serotonin that can cause carcinoid syndrome. It is believed that GI carcinoid tumors are derived from endocrine cells, regulated by ASCL-1, of the GI tract. Active Notch-1 signaling in the developing endoderm inhibits endocrine differentiation via suppression of ASCL-1. However, activation of Notch-1 signaling in the developing pancreas resulted in undifferentiated precursor cells, thereby preventing the de-

Table 1. Role of Notch-1 in various tumor types

Cancer type	Notch-1 expression level	Potential role of Notch-1
Pancreatic adenocarcinoma ^a	High	Oncogenic
Breast cancer	High	Oncogenic
Ovarian cancer	High	Oncogenic
Head and neck cancer	High	?
Cervical cancer	High	Oncogenic
Endometrial cancer	High	?
Renal cancer	High	?
Lung cancer (NSCLC)	High	?
Osteosarcoma	High	?
Mesothelioma	High	?
Glioma	High	?
Medulloblastoma	High	?
Prostate cancer	May be active	May be tumor suppressor
Papillary thyroid cancer	High	?
Follicular thyroid cancer	High	?
Small cell lung cancer ^b	Inactive	Tumor suppressor
Medullary thyroid cancer ^b	Inactive	Tumor suppressor
Pancreatic carcinoid ^b	Inactive	Tumor suppressor
Pulmonary carcinoid	Inactive	Tumor suppressor
Epithelial (skin) cancer	Inactive	Tumor suppressor
Hematologic malignancies	Inactive	Tumor suppressor

^a Blocking of Notch-1 signaling pathway inhibits growth.

^b Overexpression of Notch-1 inhibits growth.

Abbreviations: NSCLC, non-small cell lung cancer.

velopment of endocrine cells [20, 49]. In contrast to this, mice lacking HES-1, a Notch-1 effector, showed higher endocrine differentiation in the lung, stomach, and intestine [40, 50]. Recently, we have shown expression of various Notch pathway components by reverse transcription-polymerase chain reaction (RT-PCR) in GI carcinoid tumor tissues [5]. In contrast to ASCL-1 expression, other basic helix-loop-helix transcription factors such as neuro-D1 and neurogenin (Ngn-1 to Ngn-3) were not expressed in primary carcinoid tumors. Furthermore, RT-PCR reactions for various Notch receptors and ligands showed the presence of transcripts for Notch-1 to Notch-3 and DLL-1 in all carcinoid tumors tested. The human pancreatic carcinoid BON cell line also showed a detectable amount of neuro-D1, Ngn-1, and, importantly, all three Notch receptors. Interestingly, we observed the absence of active Notch-1 protein in BON cells, suggesting that the Notch signaling pathway is inactive in carcinoids. Transient expression of active Notch-1 via adenoviral vector in BON cells resulted in growth suppression and a significant reduction in NET markers such as serotonin, CgA, synaptophysin, neuron specific enolase (NSE), and ASCL-1, confirming the tumor

suppressor role of Notch-1 signaling in carcinoids [5]. Further, it was shown that the reduction in serotonin is at the level of transcription of tryptophan hydroxylase 1 mRNA, suggesting that Notch-1 signaling regulates tryptophan hydroxylase 1, a rate-limiting enzyme in serotonin biosynthesis [5]. In addition, stable expression of a Notch-1 fusion protein in BON cells also resulted in high levels of functional Notch-1 that led to an increase in the level of HES-1, an immediate downstream Notch-1 effector. Increase in the level of HES-1 significantly reduced the level of ASCL-1 protein. Similar to transient adenoviral Notch-1 activation, the stable expression of Notch-1 in BON cells also caused reductions in the levels of serotonin, CgA, NSE, and synaptophysin [2]. However, the exact mechanisms of growth and marker reduction remain unclear. Overexpression of doxycycline-inducible HES-1 in pulmonary carcinoid cells resulted in a dose-dependent growth reduction as well as ASCL-1 suppression. Interestingly, moderate growth reduction was observed with overexpression of HES-1 in carcinoid cells [3]. This indicates that there could be an additional factor(s) involved in the Notch-1 signaling pathway, mediating growth suppression. These results demon-

strate that Notch-1 pathway components are intact in carcinoid cells and that these cells are capable of responding to Notch-1 signaling. Importantly, these NET cells lack Notch-1 activation at baseline. Therefore, identification of a compound(s) that activates endogenous Notch-1 in carcinoids should be exploited. This might result in clinical applications in the treatment of patients with carcinoid disease.

MTC

MTC is derived from the calcitonin-producing thyroid C cells and accounts for 3%–5% of all thyroid cancers [4, 51]. The only curative therapy for patients with MTC is surgical treatment [52, 53]. While 80% of all MTCs are sporadic in nature, the remaining 20% are associated with germline mutations of the *RET* proto-oncogene [1, 54]. MTCs typically secrete the bioactive hormone calcitonin. In addition, MTCs express high levels of ASCL-1 and CgA. As discussed earlier, ASCL-1 is essential for C-cell development and may play a role in MTC growth and NE differentiation. Recently, we have shown that activation of the Raf-1 pathway in MTC cells by expression of estradiol-inducible estrogen receptor fused with the catalytic domain of the Raf-1 fusion protein led to complete suppression of ASCL-1 protein [6]. A decrease in the level of ASCL-1 protein correlated with a reduction in calcitonin and CgA. Furthermore, Raf-1 activation in MTC cells led to significant growth suppression [55]. To determine the role of Notch-1 signaling in MTC, we analyzed several human MTC tumor tissues and the MTC-TT cell line for the presence of Notch-1 protein and NET markers. We observed a lack of active Notch-1 protein in all tumors tested, whereas NET markers such as CgA and ASCL-1 were highly expressed [47]. Activation of doxycycline-inducible Notch-1 in TT cells by varying the concentration of doxycycline led to a dose-dependent increase in Notch-1 protein and HES-1 protein. As expected, the level of ASCL-1 was reduced with an increase in Notch-1 [47]. Further, we observed that activation of Notch-1 significantly reduced the growth of TT cells, and the reduction in growth was dependent on the level of Notch-1 protein [47]. We also found that Notch-1 regulates the calcitonin level in a dose-dependent manner. Furthermore, the levels of reduction in growth and hormone production depended on the amount of Notch-1 protein present in the cell [47]. These observations clearly support the hypothesis of the tumor suppressor role of Notch-1 signaling in MTC tumors and cell lines.

SCLC

Lung cancer is one of the leading causes of cancer-related death in the world, with almost one million deaths annually

[56]. It was estimated that 162,420 people would die of lung cancer in the U.S. alone in 2006 [57–60]. Moreover, it accounts for more deaths than prostate, breast, and colorectal cancer combined [4]. SCLC accounts for 20% of the different types of lung cancer. SCLC is extremely aggressive and is characterized by rapid growth and early metastasis. Thus, there is a pressing need for understanding the molecular pathogenesis to develop preventive or therapeutic approaches in the battle against SCLC. Similar to the observations in other tumors, such as carcinoids and MTCs, neither the Notch-1 nor the Raf-1 pathway is active in SCLC cells. In addition, these tumors express high levels of ASCL-1. Activation of the Raf-1 pathway resulted in phenotypic changes and a significant reduction in cellular proliferation [45, 61]. ASCL-1 knockout mice lack pulmonary NE cells and the pups die at birth. This demonstrates the importance of the ASCL-1 protein. Inhibition of ASCL-1 expression by antisense oligonucleotides or RNA interference has been shown to suppress the growth of SCLC cells, and the reduced expression of NET markers further supports that ASCL-1 plays a critical role in SCLC development [39, 62]. RNA interference against ASCL-1 significantly inhibited growth in both an in vitro and in vivo xenograft model [62]. It was also demonstrated that growth inhibition through suppression of ASCL-1 is mediated by cell-cycle arrest and apoptotic cell death [62]. Several studies indicated that the ASCL-1 and Notch-1 interactions are inversely proportional and that they mutually regulate the differentiation of precursors for NE cells [63–65]. It is also known that Notch-1 is a negative regulator of ASCL-1 and it is inactive in various SCLC cell lines tested [66]. Therefore, there is a high level of ASCL-1 protein in SCLC cell lines. Adenoviral-mediated expression of active Notch-1 in these cell lines resulted in both NET marker reduction and growth suppression [48]. Furthermore, the same group identified the mechanism of ASCL-1 reduction by Notch-1 activation. The reduction in ASCL-1 by Notch-1 is both at the level of transcription and post-translational degradation of the ASCL-1 protein [66]. These results further confirm that the Notch-1 pathway is not active in SCLC. Activation of Notch-1 signaling in SCLC led to growth inhibition and NET marker reduction, clearly strengthening our hypothesis of the tumor suppressor role of Notch-1 in NETs.

THERAPEUTIC APPROACHES FOR THE FUTURE

Notch-1 is a transmembrane protein, and the intracellular domain becomes active after proteolytic cleavage. It is very clear from our studies and other reports that Notch-1 signaling is absent in NETs and the activation of Notch-1 signaling by exogenous expression of active Notch-1 (NICD) results in tumor growth suppression and a significant reduc-

tion in various NET markers. Moreover, the degree of growth inhibition is directly proportional to the amount of Notch-1 present [47]. These findings indicate that activation of Notch-1 signaling may have a therapeutic role in treating NETs caused by aberrant expression of ASCL-1 and other hormones. However, aside from gene therapy, methods for the delivery of activated *notch-1* to tumor cells are nonexistent. Therefore, there is an urgent need for identifying compound(s) that activate Notch-1 signaling. In this regard, it is important to develop an assay by which Notch-1 activator can be identified using a high-throughput platform. The identification of such a compound would have a profound impact on how we treat patients with metastatic NETs. Thus far, many research groups are working on the identification of a compound that inhibits the Notch-1 pathway for other tumors such as pancreatic cancer and breast cancer. However, NET tumors clearly show the absence or inactivation of Notch-1 signaling. Based on the available data, we predict that it may be possible to activate Notch-1 in NET cells such as MTCs and GI and pulmonary carcinoids, wherein Notch-1 signaling is absent. Given the important role of Notch-1 in the regulation of growth of NETs, we hope that Notch-1-activating compounds will have novel and potent therapeutic value for the treatment of NET

patients. At this point, characterizing the levels of expression of the Notch-1 protein and also the Notch-1 pathway components in tumor tissue from patients is very important. Notch-1 and the components of this pathway could serve as prognostic markers. This will allow clinicians to tailor therapies for patients with NETs by treating them with different concentrations of Notch-1-activating compounds.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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