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The Notch signaling pathway in esophageal adenocarcinoma

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Abstract

The Notch signaling pathway plays a critical role in embryonic development, self-renewal of stem cells, and carcinogenesis. Aberrant Notch signaling has been linked to a wide variety of cancers, and can either suppress or promote tumors depending on the cell type and the context. Increasingly it is being realized that Notch signaling not only involves in the pathogenesis and development of esophageal adenocarcinoma (EAC), it also promotes the growth of EAC cells and also involved in the maintenance of EAC cancer stem cells. The efficacy of gamma-secretase inhibitor (GSI) in EAC treatment could have a major impact on easing the burden of this devastating disease. Therefore, it appears that inhibition of Notch sensitizes EAC cells to chemotherapeutic agents, which should lead to a better and more durable response to neoadjuvant chemotherapy (NAC). In this review, we bring to highlight how Notch plays a role in the development, tumorigenicity, and stemness of EAC cells, and how Notch signaling pathway could be a promising therapeutic target for the treatment of human EAC.

Key words: Esophageal adenocarcinoma (EAC), Barrett's Esophagus (BE), Notch signaling, cancer stem cells (CSCs), gamma-secretase inhibitor (GSI).

Introduction

The incidence of esophageal cancer is the eighth most common cancer in the world and sixth in cancer mortality (1, 2). Recent evidence suggests that the incidence of esophageal adenocarcinoma (EAC) now represents a significant and rapidly increasing cancer in western societies (3-6). Epidemiological data relate the increased prevalence to factors such as smoking, obesity and gastro-esophageal reflux (7-10). Esophageal adenocarcinoma is often associated with late diagnoses, poor prognoses, significant morbidities and high mortality rates (11, 12). Although the 5-year relative survival rate of esophageal cancer has been improved over the past 3 decades such as from 5% during the mid-1970s to 20% during 2004 to 2010, it is still far below the survival rate for all sites combined (13). A detailed knowledge of the molecular mechanism driving EAC would pave the way for developing more effective treatment strategies for EAC that would ultimately improve patient outcome.

Research over the years has gradually and sequentially brought Notch signaling pathway into the limelight of the cancer research. It is now well accepted that Notch plays a critical role in embryonic development and self-renewal of stem cells, and functions as an oncogene in many human neoplasms (14, 15). Notch is aberrantly re-activated and can transform epithelial cells in culture and drive tumorigenesis in mouse models (16). Binding of the Notch ligand renders the receptor susceptible to proteolytic cleavage mediated by an intramembrane protease complex termed γ-secretase (17-19), which in turn results in the release of the Notch intracellular domain (NICD) and its recombination associating with DNA-binding protein in the nucleus. The efficient γ-secretase inhibitors have been developed to explore the effects of loss of Notch signaling (20-23). The first evidence implicating Notch signaling in human cancer came from a chromosomal translocation of the mammalian *NOTCH1* gene that resulted in constitutive activation of Notch signaling in a subset of T cell acute lymphoblastic leukemia (T-ALL) (24, 25). Subsequently, it was found that about 50% of the cases of T-ALL harbor activating mutations in the *NOTCH1* locus (26). It is clear that Notch functions as an oncogene, as it can transform epithelial cells in culture and drive tumorigenesis in mouse models (16, 27). Notch signaling has been observed in a wide variety of cancers, including breast cancer (28), colorectal cancer (29), pancreatic cancer (30), small-cell lung cancer (31) and cervical cancer (32). Data derived from these different tumor types suggests that Notch signaling affects multiple aspects of the cancer phenotype (33, 34)(Table 1). Given that Notch has been shown to regulate the self-renewal properties and the differentiation status of many cell lineages, including embryonic stem cells, the notion that Notch signaling has a possible role in promoting self-renewal of the cancer stem cells (CSCs) is attractive (35, 36). This concept has, in recent years, begun to emerge as the Notch pathway has increasingly been associated with these phenotypes in cancers of various origins.

In this review we partition the content into the role of Notch signaling in the multistep developmental process of EAC, the responsibility for tumor recurrence, metas-

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Table 1. Role of Notch signaling in esophageal cancer and other human solid tumors.

	Oncogenic	Tumor suppressive	Tumor progression	Tumor maintenance	Drug resistance
Esophageal Adenocarcinoma	(37)		(37, 38)	(38, 39)	(38)
Esophageal Squamous Cell Carcinoma	(40)		(41-46)	(47)	(46)
Breast Cancer	(48)	(48)	(28, 49-51)	(49)	(33)
Cervical cancer			(32, 52-54)		(55)
Colorectal cancer	(56-58)				(34, 59)
Head and neck Cancer					(60)
Lung Cancer	(31)	(61)	(31, 62-64)		
Medulloblastoma			(65, 66)	(66)	
Melanoma	(27, 67-69)			(68, 69)	
Pancreatic Cancer	(30, 70-72)		(30, 73-75)		(76-79)
Skin Cancer		(80, 81)			

tases & chemo-resistance, and the potential improvement in patient outcome by targeting Notch for EAC.

Notch Signaling in Barrett's Esophagus (BE)

The Notch signaling pathway plays an important role in embryonic development and self-renewal properties of the stem cell in gastrointestinal tract. It had been demonstrated that canonical Notch signaling is activated in the stem or progenitor domain of gastrointestinal epithelium in basal layer of esophagus mucosa, as well as in lower part of the colon crypt (82). EAC is thought to arise through multiple stages of carcinogenesis, including the replacement of the normal squamous epithelial lining with a columnar intestinal metaplasia, termed Barrett's esophagus (83, 84). BE is a pre-neoplastic metaplasia in which the normal squamous epithelia of the distal esophagus is replaced with intestinal mucin-producing goblet-like cells. Preliminarily, a study implied the aberrant activation of Notch involved in the mechanism of BE conformation (85). Moreover, an earlier study found the relationship between the Notch pathway and Cdx2 expression in the development of BE. They forced the interrelationship between expression of Cdx2 and Notch target gene Hes1, and indicated that Cdx2 enhanced by stimulation with bile acids may induce specialized intestinal metaplasia (SIM) by regulation with Notch pathway (86). Furthermore, a recent study has revealed that upregulated transdifferentiation of the esophageal epithelial cells by Notch signaling inhibition can promote BE-like metaplasia in part via upregulation of KLF4 (87).

Progression of disease then follows through a dysplastic phenotype followed by adenocarcinoma. The estimated rate for a patient to progress from Barrett's to adenocarcinoma is roughly 0.3-0.5 % per patient year (88). The mechanistic details have not yet been fully described for this stepwise progression of esophageal disease with respect to driver and passenger mutations. The Notch pathway is an evolutionarily conserved signaling mechanism between adjacent cells that plays a critical role in development and self-renewal of stem cells (89). Furthermore, since expression of the Notch pathway in the adult is largely restricted, it has become an attractive target for therapeutic intervention. The general scheme for Notch signaling is that ligands presented on adjacent cells initiate the Notch activation cascade leading to the cleavage and release of Notch from the plasma membrane. Following this cleavage, which is the result of the presenilin-gamma secretase complex, NICD translocates into the nucleus (90). Once in the nucleus Notch initiates and maintains a transcriptional cascade to drive the physiological response. Notch expression has been noted in BE and inhibition of Notch by GSI treatment reduced the cellular differentiation of goblet cells in two BE animal models (39, 91). However, it is unclear whether Notch itself is driving either BE or the neoplastic conversion of BE to frank adenocarcinoma, given that Notch expression can be seen in normal cells of the intestinal mucosa and gastric fundus. That is, inhibition of Notch in this experimental system could simply be altering the differentiation of progenitor cells. Furthermore, it is not clear if Notch expression in itself predicts progression or status of disease, as the association of Notch expression to progression of BE is not concordant.

Notch Signaling in Esophageal Adenocarcinoma

Accumulating evidence for the inhibition of Notch signaling in the differentiation of secretory cells came from gastric cancer and colorectal cancer (59, 82). However, the opposing anti-oncogenic roles of Notch signaling also have been revealed in esophageal squamous cell carcinoma (SCC) by promoting keratinocyte differentiation (82). The anti-cancer activities of Notch inhibitor GSI had been disclosed in models of gastric cancer and colorectal cancer (59, 82). The presence of activation of Notch signaling was observed in metaplastic BE epithelium and two well-known human Barrett's-derived EAC cell lines, OE33 and SKGT-5, but not in the normal human esophagus (39). It had been demonstrated that Notch and transforming growth factor β (TGF-β) signaling pathways play important roles in regulating self-renewal of stem cells, cell-fate determination and gastrointestinal carcinogenesis(35, 37). Mendelson et al. demonstrated that levels of Notch signaling components Hes1 and Jagged1 dramatically

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were raised in EAC tumors and cell lines compared to normal tissues, while loss of Smad4 and β2 spectrin (β2SP) in BE and EAC (35). Similarly, Song et al. indicated that Notch signaling and its target SOX9 could be activated by suppression of TGF-β activity via loss of TGF-β adapter β2SP (SPNB2) in EAC (37).

Recently our study provided compelling evidence showing that the Notch pathway plays a critical role in regulating the growth and maintenance of esophageal adenocarcinoma (38). Detailed analysis indicated that the aberrant activation of Notch signaling involves in human EAC, and the expression of activated Notch1 was also found to be associated with the differentiation grade, stage and lymph node (LN) metastasis of EAC (38). The earlier studies only evaluated activity of Notch signaling in normal esophagus, BE, and EAC cell lines (35, 39)The status of Notch signaling had been investigated in surgically resected EAC tumors and demonstrated that a significant majority of samples exhibit increased Notch activity as compared to adjacent normal tissue in this study (38). There is a direct piece of evidence suggesting that the elevated Notch activity was associated with the chemoresistance of EAC that is poorly differentiated tumors have greater Notch activity, and the expression level of NICD, and Notch target gene transcription were elevated in the tumors derived from chemo-resistant cases (38). Blocking Notch activity by GSI affects the proliferation and/or survival of EAC cell lines and human EAC samples in *ex vivo* cultures. In contrast, this inhibition was not observed in human esophageal epithelial cell line, Het-1A (38). Since clinical EAC survival data also implicated that higher Notch activity correlates with poor prognosis and considering the high degree of Notch expression in samples derived from patients that failed neoadjuvant chemotherapy (NAC), spotlight was set on if there is a different role for Notch in advanced and developing tumors, and whether Notch activity was the cause of resistance to NAC (38).

To answer the question whether resistant cells were selected by treatment with NAC or does Notch activity in the tumor confer resistance, esophageal ultrasound-assisted endoscopic biopsies obtained from patients prior to treatment with NAC at the time of staging were analyzed for activated Notch. It was revealed that that a patient with no detectable activated Notch had a complete pathological response to NAC whereas two patients who had detectable levels of activated Notch had only a limited response (38). Although the number of the cases examined is limited, the result was consistent with the most observations and hypothesis. Data obtained through a variety of different technologies has shown that Notch confers resistance to chemotherapy. Furthermore, studies have demonstrated that Notch signaling was indispensible for the cellular proliferation and tumor growth of EAC by a series of experiments *in vitro* and *in vivo.* In accordance with this approach, in three patient-derived xenografts (PDX) models showed that inhibition of Notch activity dramatically reduced the growth and size of tumors (38). This is particularly significant in light of the fact that these patient derived tumors are more representative of the genetic diversity of the patient population than are tumor derived cell lines, and could predict the efficacy of targeted therapies. Taken together, these data strongly support the use of GSI in the clinical management of EAC in combination with standard-of-care (SOC) chemotherapy regimens.

Notch Signaling in Esophageal Adenocarcinoma Cancer Stem Cells

There is increasing evidence that cells within a tumor can exhibit heterogeneity. Cancer stem cells (CSCs) are thought to be important for driving tumor development and to be responsible for many attributes of cancer that drive mortality (92, 93). These CSC sub-populations have been implicated in the resistance to chemo- and radiation-therapy, metastasis and relapse of disease (94, 95). Although this concept remains theoretical, subpopulation of cells have been derived from tumors that have distinct characteristics for proliferation, sensitivity to therapy and tumor initiation (96, 97). Esophageal adenocarcinoma is often considered to arise from a clonal stem-like population of cells, which is potentially responsible for its poor prognosis (35).

Accumulating evidence suggests that Notch signaling plays an important role in cancer stem cells as mentioned above. The cancer stem-like cells or side population (SP) cells were isolated and identified in human esophageal squamous cell carcinoma (ESCC) cell lines, EC9706 and EC109, using Hoechst 33342. Those "Tip"-SP cells showed increased levels of Notch and WNT signal pathway components, such as FZD10, PTGS2, KLF5, TTK, and RBM15 (98). A xenograft experiment in which viable tumor cells were isolated from mice harboring EAC tumors treated with either Notch inhibitor DAPT or vehicle DMSO. Cells derived from the mouse treated with DAPT, although viable, failed to form tumors when transplanted into naïve mice, whereas cells derived from the DMSO treated mice readily formed tumors. Indicating that inhibition of Notch signaling stunts xenograft proliferation and completely eradicates a specific sub-population of cells within the tumors that are capable of forming secondary tumors. On the other side, Notch signaling appears to drive chemoresistance in EAC (38). Collectively, these findings suggest that Notch signaling regulates the cancer stem cell population. Therefore, Notch appears to be driving the cancer stem cell phenotype by initiating a transcriptional program that establishes and maintains stemness in EAC.

Although extensively studied, the CSC surface marker had been well known in a number of cancers, it is still largely debated and no reliable surface markers for isolating and identifying EAC CSCs. The common CSC surface makers, such as CD44, CD24, EpCAM, CD133, CD184, and CD71, are not helpful to isolate the subpopulation of EAC CSCs (99). Instead, the sphere culture and aldehyde dehydrogenase (ALDH) assay had been used in most studies of CSCs, which characterize stemness of EAC cells *in vitro* and *in vivo*. The tumorspheres grown under serum-free, low attachment conditions have shown they are enriched for specific stem-like characteristics, and have emerged as one among the most widely studied approach in cancer stem cell research (100). The EAC tumor spheres had higher levels of Notch activity, and lost their spheres forming ability

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after GSI treatment (38). As a true marker of the enrichment of tumor-forming cells, the EAC tumor spheres were at least 10 times more efficient in forming tumor xenografts. In contrast, analysis of ALDH activity could clearly distinguished populations of cells that were enriched in their ability to form spheres in cultures. Indeed, not only did ALDH positive cells more readily form tumor spheres but they also had a greater degree of activated Notch signaling and were sensitive to DAPT treatment (38). In fact, pre-treatment of adherent cells with GSI significantly reduced the number of ALDH+ cells and resulting in the formation of spheres, indicating that Notch is critical in the subpopulation of adherent cells that give rise to spheres.

To further characterize the EAC tumor spheres as a bone fide population of CSC, the expression of a set of CSC signature genes were compared between adherent EAC cells and tumor spheres. This set of genes are including cell surface markers, enzymes and transcription factors associated with the less differentiated, tumor forming, cancer stem cell phenotype (101). Higher levels expression of these genes accompanied elevated Notch activation in spheres (38). Furthermore, inhibition of Notch signaling by DAPT treatment of EAC tumor spheres significantly reduced the expression of a subset of these cancer stem cell marker genes including *ALDH, CD133, CD24, SOX2, TWIST1* (38). The other genes examined although induced by what appears to be a Notch directed program but they did not display the kinetics of a direct transcriptional target. Song et al. reported the levels of SOX9 was elevated markedly in EAC tumor tissues compare to normal tissues, and they demonstrated the correlation of SOX9 expression and some facts of tumor progress, such as poorer survival and lymph node invasion in EAC patients (37). Emerging data and the information suggests that activation of Notch signaling by Notch1 could promote colony formation and increased expression of SOX-2 and OCT-4 in Flo-1 and Het-1A cells. Spheres from EAC cell lines shows characteristics of cancer stem cells such as elevated levels of SOX-2 and OCT-4 and increased tumor growth efficiency in xenografts. Detailed mechanistic insights highlighted that SOX-2 was identified as a direct target of Notch1 in EAC cells by Chromatin Immunoprecipitation (ChIP) studies. In contrast, *OCT4* is not a direct target of Notch in these EAC tumor spheres as Notch did not directly induce *OCT4* transcription nor could it be localized by ChIP on the *OCT4* promoter. Therefore, considering this specificity, one could postulate that the other markers that respond to DAPT treatment are likewise direct targets.

Notch Inhibitors for Clinical Management of Esophageal Adenocarcinoma

The early evidence for effect of Notch inhibition came from a systemic treatment with Notch inhibitor GSI in a well-validated rodent model for BE. Menke *et al.* reported that Notch inhibition promotes the proliferative BE cells convert to differentiated goblet cells, and implied a novel therapeutic strategy, that is GSI could be applied as a local treatment to improve this increasingly common pre-malignant condition (39).

The anti-cancer efficacy of suppression of Notch activity by GSI in EAC had been disclosed by a series of experiments in vitro and in vivo, by significantly decreasing cell proliferation and downsizing tumor growth (38). Activation of Notch pathway was elevated in clinical samples from chemo-resistant EAC cases with chemotherapy resistance, appears that Notch signaling is driving resistance to chemotherapy by maintaining a robust population of CSC. Suppression of Notch activity by GSI is efficacious in increasing sensitivity of OE33 cells to 5-FU treatment, also indicated that that inhibition of Notch depletes the CSC population and sensitizes cells to chemotherapeutic agents which should lead to a better and more durable treatment response to NAC. It was experimentally verified that EAC cells with higher levels of Notch signaling are more resistant *in vitro* to 5-FU, a commonly used drug in the treatment of EAC (38). Interestingly, contemporary study indicated that EAC cells originally with low level of Notch activity are sensitive to 5-FU and can be converted to resistant cells by ectopic expression of Notch1 (38). Data presented herein strongly suggest that Notch signaling drives a significant proportion of esophageal adenocarcinomas. Notch signaling does so by establishing and maintaining a cancer stem cell like population of cells, which also underlies resistance to chemotherapy.

Although some studies demonstrated a potential improvement in outcome of EAC patients by Notch signaling pathway, the results of long-term treatment with GSIs seems to be highly context dependent due to the inadvertent effects on other developmental pathways. The evidence of positive regulation is Notch signaling could base on the concomitant loss of β2SP/TGF-β in EAC (35). GLI1/Hedgehog signaling transcription is upregulated in a variety of human tumors, such as basal cell carcinoma, lung cancer, breast cancer, gastric cancer, pancreatic cancer, and esophageal cancer (102). Notch-CSL-HES/HEY downregulates GLI1 transcription in medulloblastoma and glioblastoma (GBM) cells, which involves with activation of multiple developmental pathways including Notch, Hedgehog, and WNT/beta-catenin (102, 103). Furthermore, resistance of long-term GSI treatment has been observed by concomitant upregulation of WNT activity in GBM cells (103). It has been indicated that GLI1/Hedgehog signaling transcription is upregulated in EAC and HH activation is involved in the development of EAC (104). Moreover, an earlier study reported that the WNT/beta-catenin pathway implicates in the carcinogenesis of BE (105). There is a potential mechanism of therapeutic resistance for application of Notch inhibitors via possible compensate for therapy by upregulating other developmental pathways.

As a consequence, the possibility was raised that the effect of combined therapy targeting multiple pathways simultaneously might inhibit tumor growth more effectively than monotherapy. The dramatically increased efficacy from treatment targeting Notch and Hedgehog simultaneously has been demonstrated in inducing apoptosis, decreasing cell growth, and inhibiting colony-forming ability compare to monotherapy in GBM and medulloblastoma cell lines and primary neuro-spheres isolated from human GBM tumor samples (103). It is still not clear how about the crosstalk between Notch pathway and other developmental pathways, and the co-

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inhibition of the combinational treatment with multiple inhibitors in EAC cells.

Conclusion and Perspectives

Pharmacological inhibitors of Notch had been applied as anti-cancer drugs in clinic trials, and potential therapeutic value of Notch inhibitor alone or in combination with chemotherapeutics has been clinically evaluated on some cancer for breast cancer, pancreatic adenocarcinoma, gastric cancer, and colorectal cancer, etc. (82, 106-110). Notch signaling pathway is critical for EAC and underlies resistance to chemotherapy. Particularly, the regulation of Notch signaling in cancer stem cell phenotype of EAC is worth the whistle. Given that inhibition of Notch by GSIs is efficacious to sensitize EAC cells to chemotherapeutic agents, one might expect that blockage of Notch might lead to a better and more durable response to neoadjuvant chemotherapy and therefore improve the clinical management of EAC. Although data released strongly suggest that Notch signaling drives a significant property for establishing and maintaining of CSCs, which also underlies resistance to chemotherapy, it is imperative to discuss how is the detailed knowledge of the molecular mechanisms driving EAC stemness and progressing. Even the studies lay the foundation for a clinical trial looking at the efficacy of GSI in EAC treatment, the question is still unsolved whether targeting multiple developmental pathways can be more effective than monotherapy at EAC cells. Moreover, the significance of Notch activity in patients' biopsy for predicting outcome to chemotherapy also need more appropriate EAC cases to identify. As a hopeful treatment strategy, development of Notch-targeting agents is continuing continually. Taken together, it would be a useful strategy and potential improvement to target Notch signaling pathway either using single reagent or combining with certain typical chemotherapeutics or/and other developmental pathways' inhibitors to ultimately provide a more durable cure to this disease.

Other articles in this theme issue include references (111-122).

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