Notch Signaling Drives Stemness and Tumorigenicity of Esophageal Adenocarcinoma

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Abstract

Esophageal adenocarcinoma ranks sixth in cancer mortality in the world and its incidence has risen dramatically in the Western population over the last decades. Data presented herein strongly suggest that Notch signaling is critical for esophageal adenocarcinoma and underlies resistance to chemotherapy. We present evidence that Notch signaling drives a cancer stem cell phenotype by regulating genes that establish stemness. Using patient-derived xenograft models, we demonstrate that inhibition of Notch by gamma-secretase inhibitors (GSI) is efficacious in downsizing tumor growth. Moreover, we demonstrate that Notch activity in a patient’s ultrasound-assisted endoscopic–derived biopsy might predict outcome to chemotherapy. Therefore, this study provides a proof of concept that inhibition of Notch activity will have efficacy in treating esophageal adenocarcinoma, offering a rationale to lay the foundation for a clinical trial to evaluate the efficacy of GSI in esophageal adenocarcinoma treatment. Cancer Res; 74(21); 6364–74. ©2014 AACR.

Introduction

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death in the world (1). The incidence of esophageal adenocarcinoma, a subtype of esophageal cancer, has been on the rise in the United States and other Western countries over the past 30 years (2–4). The prognosis for esophageal adenocarcinoma is poor, with a 5-year survival rate of 19% (5) and only 0.9% for advanced esophageal adenocarcinoma (6). Currently, a detailed molecular understanding of the underlying pathophysiology of esophageal adenocarcinoma has not been realized. However, it is generally accepted that the etiologic condition that drives the onset of esophageal adenocarcinoma is gastrointestinal reflux disease followed by a metaplastic condition termed Barrett esophagus (7, 8). Although the link is compelling, only a small percentage of patients with Barrett esophagus progress to frank adenocarcinoma (9). Similarly, the molecular details that drive progression from Barrett esophagus to esophageal adenocarcinoma remain poorly understood. Therefore, it is imperative that a detailed knowledge of the molecular mechanisms driving esophageal adenocarcinoma is obtained to develop more effective treatment strategies and improve clinical management of esophageal adenocarcinoma.

Given that Notch acts as an oncogene and is aberrantly re-activated in many human neoplasms, we sought to determine a role for Notch in esophageal adenocarcinoma (10, 11). Herein, we present evidence that Notch signaling is greater in the less differentiated tumors and drives a cancer stem cell phenotype. We demonstrate that Notch signaling is critical for these esophageal adenocarcinoma cancer stem cells (CSC) and regulates genes that establish stemness. Using patient-derived xenograft models, we demonstrate that inhibition of Notch signaling by gamma-secretase inhibitors (GSI) is efficacious in downsizing tumor growth. Moreover, we provide evidence that demonstrates Notch activity in a patient’s ultrasound-assisted endoscopic (EUS)–derived biopsy sample can predict outcome to chemotherapy. Therefore, it appears that Notch signaling is driving resistance to chemotherapy by maintaining a robust population of CSC and that inhibition of Notch depletes the CSC population and sensitizes cells to chemotherapeutic agents, which should lead to a better and more durable response to neoadjuvant chemotherapy (NAC).

Materials and Methods

Human esophageal adenocarcinoma and normal esophageal mucosa samples

Human esophageal adenocarcinoma tumors, matched adjacent nontumor tissues, and normal esophageal mucosa were obtained from tissue microarray (Biomax.US; ES8011)
and the patients undergoing surgery at the Miller School of Medicine, University of Miami. We obtained consent from all patients and approval from the Institutional Research Ethics Committee.

Cell culture

Human esophageal adenocarcinoma cell lines OE33 and OE19 were obtained from the European Collection of Cell Culture. FLO1 and JH-EsoAd1 cells were a generous gift from other laboratories (see Acknowledgments). Normal human primary esophageal epithelial cells EAC09N, EAC10N, and EAC11N were isolated from human esophageal mucosa obtained from normal adjacent tissue. Het-1A, a human immortalized esophageal epithelial cell line, was obtained from ATCC. All cell lines were characterized by short tandem repeat analyses (STR) profiling (LGS Standards SLU) within 6 months after receipt.

Immunohistochemistry and immunofluorescence

Immunohistochemical staining of Notch intracellular domain (NICD; 1:200; ab-8925) and DLL4 (1:200; ab-7280) were carried out using a Dako autostainer. Anti-rabbit IgG labeled with red-fluorescent Alexa Fluor594 dye (1:200; Invitrogen A21207) was used as the secondary antibody for immunofluorescence.

Quantitative RT-PCR

Total RNA was isolated and cDNA was synthesized according to the manufacturer’s protocol. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermal cycler conditions were 50°C for 2 minutes and 95°C for 10 minutes, then 40 cycles of 15 seconds at 95°C (denaturation) followed by 1 minute at 59°C (annealing and extension; ref. 12). GAPDH was used to normalize gene expression. All samples were normalized to the relative levels of GAPDH and results were expressed as fold increase in relative levels of all.

Western blotting

Cells lysates were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked in milk and incubated with the antibodies followed by incubation with the anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase. For detection, enhanced chemiluminescence reaction (Amersham Biosciences) was done according to the manufacturer’s specification.

Lentiviruses and infection

Lentiviruses expressing various shRNAs and overexpression plasmids were produced as described previously (13). For viral infection, subconfluent cells were overlaid with the virus-containing medium and fresh growth medium in the presence of polybrene (Sigma).

Luciferase assay

Cells grown in 24-well plates were transiently transfected with CSL/GFP reporter plasmid using Lipofectamine 2000 (11668-019; Invitrogen) and luciferase activity was measured in cell lysates after 24 hours.

Colony formation assay and cell viability assays

Cells were cultured at low density under treatment, and then colonies were stained with 0.01% crystal violet and counted. The cells were measured using the Cell Titer-Glo assay (G7572; Promega) for cell viability assays.

Tumor sphere formation assay

To obtain tumor spheres, cells were cultured in DMEM/F12 with 2% B-27 serum-free supplement (17504-044; Invitrogen), 20 ng/mL epidermal growth factor (EGF; PHG0311L; Invitrogen), and 20 ng/mL basic fibroblastic growth factor (FGF; PHG0266; Invitrogen) for 14 days to select for CSCs and early progenitor cells. Resulting tumor spheres were examined and counted under the microscope.

Flow cytometric analysis of aldehyde dehydrogenase

Cells were stained using the ALDEFLUOR Kit (STEMCELL Technologies) following the manufacturer’s instructions and were analyzed by flow cytometry, as described previously (14).

Chromatin immunoprecipitation assay

OE33 and FLO1 cells were cross-linked with 1% formaldehyde and cross-linking was quenched by adding glycine to a final concentration of 0.125 mol/L. Cells were resuspended in SDS lysis buffer and sonicated to yield chromatin fragments of approximately 300 to 800 bp. Lysates were immunoprecipitated with α-Notch 927 (polyclonal), α-Notch (ab27526; Abcam), or α-Pragmin (Bethyl Laboratories) antibodies and were reverse cross-linked at 65°C in 200 mmol/L NaCl for 4 hours followed by incubation with RNase A and proteinase K. DNA was cleaned using the PCR Purification Kit (Qiagen) and Sox2 and GAPDH were amplified by qPCR. Primer sequences are available upon request.

Animal experiments

Six-week-old SCID/hairless mice and CD-1 Nude mice were purchased from Charles River Laboratory, and NOD-SCID gamma (NSG) mice from The Jackson Laboratory. Animal experiments were approved by the University of Miami Institutional Animal Care and Use Committee. Esophageal adenocarcinoma cells were injected subcutaneously. When the tumor size reached 200 mm3, the mice were split into two groups uniformly. Patient-derived xenograft (PDX) cancer models were established as described previously (15) in NSG mice. Tumor volume was measured by the formula: volume = (S × S × L)/2 (15). The xenografts were harvested and samples were subjected to histologic examination.

Genome-wide expression meta-analysis

The genome-wide expression data from 64 patients with esophageal adenocarcinoma using Illumina human-6 v2.0 expression microarrays (Illumina) were obtained from NCBI Gene Expression Omnibus (GEO) database (GEO accession number: GSE13898; ref. 16). The 64 patients with esophageal
adenocarcinoma were divided according to their expression pattern using an unsupervised hierarchical clustering analysis as previously described (16). Expression analysis was performed to compare the gene expression profile on the 64 esophageal adenocarcinoma samples using the Agilent Gene-Spring software v12.0 (Agilent Technologies). Significant differences in gene expression were determined by the Student t test. The p values were further adjusted for multiple comparisons using the Benjamini–Hochberg FDR multiple testing correction, and was set at 0.05.

Statistical analysis

P value was calculated using the χ² in contingency table. Data are presented as mean ± SD and were analyzed by the 2-tailed Student t test. A P value of less than 0.05 was considered significant. Enhanced expression of NICD in esophageal adenocarcinoma tumors versus normal mucosa was determined by the Mann–Whitney U test. In all other cases, statistical significance was determined by the Student t test. P < 0.05 was considered statistically significant.

Results

Elevated Notch activity is associated with the differentiation state and clinical stage of esophageal adenocarcinoma, drives resistance to chemotherapy, and results in poor prognosis

To assess the status of the Notch pathway in esophageal adenocarcinoma, we screened primary esophageal adenocarcinoma samples for presence of NICD and expression of Notch target genes. NICD was present in 72.5% (29 of 40) of primary esophageal adenocarcinoma tumor tissues via immunohistochemistry (IHC; Fig. 1A).

Figure 1. Notch activity elevated in esophageal adenocarcinoma (EAC) and associated with the differentiation state and clinical stage. A, NICD staining was shown in human esophageal adenocarcinoma and normal esophageal mucosa tissues. B, level of NICD was detected by Western blotting in esophageal adenocarcinoma and adjacent normal mucosa. C, mRNA levels of Notch targets were determined by qPCR and were normalized to the relative expression values of matched adjacent normal mucosa (set to 1). Error bars, ± SEM. D, hematoxylin and eosin (H&E) and IHC staining of NICD were determined in the esophageal adenocarcinoma with well-, moderately, and poorly differentiated tumors. E, percent positive of NICD staining in the esophageal adenocarcinoma with different clinical stages (***, P < 0.0001). F, percent positive of NICD staining in the esophageal adenocarcinoma with different clinical stages (***, P < 0.0001). See also Supplementary Figs. S1 and S2.

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of the normal esophageal mucosa (Fig. 1A) and in cells of the normal gastric cardia (Supplementary Fig. S1C). Similarly, Western blot analysis of primary tumors and normal tissue displayed an increase in NICD expression (Fig. 1B). The mRNA levels of Notch target genes (HES1, HEY1, HEY2, and HEYL) and Notch ligands (JAG1, JAG2, DLL1, DLL3, and DLL4) were also elevated in tumor samples compared with normal tissue (Fig. 1C, Supplementary Fig. S1A). We observed a similar increase in NICD and a commensurate increase in Notch target gene expression in esophageal adenocarcinoma cell lines as compared with normal cells (Supplementary Fig. S2). When we compared the levels of NICD in esophageal adenocarcinoma tumors relative to their stage and degree of differentiation, we find that high levels of NICD were observed in 94.4% (17 of 18) of the poorly differentiated esophageal adenocarcinoma tumor cases, whereas fewer and weaker positive nuclear staining for NICD was observed in 54.5% (12 of 22) of the well or moderately differentiated esophageal adenocarcinoma cases (P < 0.0001; Fig. 1D and E). This indicates that Notch activity correlates with the stage of esophageal adenocarcinoma (Fig. 1F).

Current treatment guidelines for esophageal adenocarcinoma include NAC followed by surgical resection. Patients that had a complete pathologic response to NAC exhibited a significantly greater 5-year survival compared with patients with esophageal adenocarcinoma who did not have a significant response. However, only 16% of patients experienced a complete pathologic response. The other 74% of patients with esophageal adenocarcinoma either had no response or partial response to NAC therapy (17–19). We sought to determine if Notch played a role in the response to NAC. Therefore, we analyzed a set of 28 surgically resected esophageal adenocarcinoma samples derived from patients that failed NAC therapy for activation of the Notch pathway by IHC and qPCR. Although Notch activity was elevated in the untreated group, the tumors that showed only a partial response, or were refractory to treatment, had appreciably greater levels of Notch signaling (Fig. 2A–C). This indicates that tumors with elevated Notch activity may have been selectively enriched by chemotherapy or may be resistant to chemotherapy. To gain insight to this issue, we analyzed the expression of NICD in chemo-naive esophageal adenocarcinoma samples derived from EUS biopsies. Consistent with our hypothesis, a patient sample that had undetectable levels of NICD had a complete response to chemotherapy, whereas two patients that had high levels of activated Notch did not show significant response to chemotherapy (Fig. 2D). Therefore, activated Notch seems to predict response to chemotherapy. Consistent with these results, an analysis of gene expression data from the GEO indicates that activation of Notch signaling is associated with poor prognosis in esophageal adenocarcinoma (Table 1).

**Notch activity promotes the proliferation and/or survival of esophageal adenocarcinoma cell lines in vitro**

To assess the role of Notch signaling in cell proliferation and survival, we blocked the Notch pathway by treating cells with DAPT, a commonly used GSI. Efficacy of the treatment was demonstrated by a dramatic reduction of NICD as seen by immunofluorescence in OE33 cells (Fig. 3A). There was also a reduction in Notch-mediated transcription as seen by luciferase reporter activity and a decrease in transcription of Notch target genes (Fig. 3B and C). Treatment of esophageal adenocarcinoma cell lines with DAPT caused a decrease in cell viability (Fig. 3D). The number and size of colonies formed by OE33 and JH-EsoAd1 cells treated with DAPT were significantly lower than those from mock treated cells, suggesting that the reduction in proliferation is due to loss of Notch signaling. Inhibition of the Notch pathway did not alter the proliferation of Het-1A cells (Fig. 3E, bottom). To further validate this observation, and to rule out off-target effects of GSI, we knocked down the expression of CSL via shRNA (80%–85% reduction; Fig. 3F). There was a significant reduction in the proliferation and colony formation of OE33 cells with CSL knockdown compared with control cells (Fig. 3G and H). Together, these results suggest that the Notch pathway is required for proliferation and survival of esophageal adenocarcinoma cells in vitro. Conversely, an increase in Notch signaling had the opposite effect. We established Het-1A/NICD and FLO1/NICD cell lines with stable expression of NICD. Ectopic activation of Notch pathway was confirmed by qPCR and immunoblotting (Supplementary Fig. S3). Het-1A/NICD and FLO1/NICD cells formed more colonies as compared with control cells harboring an empty vector (Supplementary Fig. S3C and S3F), suggesting that increased activation of Notch signaling promoted the transformation of Het-1A cells and proliferation of FLO1 cells. Similar results were observed when exogenous NICD was expressed in OE33 cells (Supplementary Fig. S3G). Collectively, these data indicate that NICD is a critical regulator of esophageal adenocarcinoma cell proliferation and transformation of normal esophageal epithelial cells in vitro.

**Suppression of Notch activity inhibits tumor growth in esophageal adenocarcinoma xenograft models**

To test the effect of Notch signaling on the growth of esophageal adenocarcinoma cell lines xenografts, OE19 and OE33 cells were injected into the flank of immunocompromised mice. Once tumors reached 200 mm³, we treated the animals with DAPT (20 mg/kg) via daily intraperitoneal injections. Tumor growth was significantly stunted in the DAPT treatment group as compared with vehicle group for both cell line xenografts (Fig. 4A and B). Compared with control, DAPT treatment reduced expression of the Notch target gene HES1 (P = 0.0119; Fig. 4C) and a decreased the level of NICD (Fig. 4D). DAPT treatment also caused a decrease in proliferation and an increase in apoptosis as measured via Ki67 staining and TUNEL assay, respectively (Fig. 4D). Furthermore, treatment completely inhibited the growth of OE19 cell xenografts when treatment was initiated 1 day following cell transplantation (Fig. 4E). These data indicate that Notch activity is indispensable for tumor establishment and maintenance.

Because we observed that Notch activity was required for the initiation of xenograft tumor formation, we wanted to...
determine if the residual tumor cells in a xenograft tumor treated with DAPT could reestablish a tumor. To address this point, OE19 xenograft tumors were established and treatment was initiated as previously described for 2 weeks. We observed that tumors treated with DAPT were significantly reduced in size compared with the control group (Fig. 4F). Following treatment, tumors were harvested and cell suspensions were prepared. Both mock and DAPT-treated cell suspensions contained approximately 80% viable cells as determined by trypan blue exclusion. A total of 1 x 10^6 viable cells from each treatment group were then transplanted into both flanks of five nude mice. Mock treated cells developed tumors, whereas DAPT-treated cells failed to develop any noticeable tumors 10 weeks after reimplantation (Fig. 4F). The remaining cells, we postulate, are comprised of bulk tumor cells that evidently lack the ability to reestablish a tumor in mice. Therefore, inhibition of Notch signaling selectively abolished the subpopulation of cells capable of forming new tumors (i.e., tumor-initiating cells or CSCs) in the xenograft.

We sought to extend our findings using PDX. PDX models better represent the diversity of human cancer compared with cell line–based xenografts and are more representative of the original tumor. Therefore, the therapeutic efficacy in PDX models is a better predictor of the clinical response of the patient’s tumor from which the model is derived. Three PDX models were established from patients with esophageal adenocarcinoma, and these tumors display the same histologic and IHC characteristics as the primary tumors (Fig. 5A). Similar to cell line–based xenografts, inhibition of Notch signaling by DAPT in the PDX model led to significantly stunted growth and a reduction in proliferation and increased apoptosis as compared with the vehicle group (Fig. 5B and C).

Taken together, these data clearly demonstrate a critical role...
for Notch signaling in the proliferation and development of esophageal adenocarcinoma tumors.

**Esophageal adenocarcinoma–derived tumor spheres display characteristics of CSCs**

The experiment shown in Fig. 4F indicates that inhibition of Notch signaling in the xenograft models is selectively targeting the CSC population of the tumor. Therefore, we sought to examine the role of Notch on the CSC population in esophageal adenocarcinoma. To address this, a sphere-culture system was adopted for the isolation/enrichment of esophageal adenocarcinoma CSCs. Several publications have shown that cells grown under serum-free, low attachment conditions are enriched for specific stem-like characteristics (20). Esophageal adenocarcinoma cell spheres were obtained from cell lines after culture as outlined in Materials and Methods (Supplementary Fig. S4A). We were able to obtain cell spheres from all the cell lines tested. A cells ability to form spheres was relative to the level of NICD present in the attached culture. OE33 cells have more NICD under attached conditions and are more efficient in forming spheres (Fig. 6A and Supplementary Fig. S4B). When compared with attached cells, all esophageal adenocarcinoma cell line spheres had greater levels of NICD expression. To validate that esophageal adenocarcinoma cell line sphere cultures were indeed enriched for CSCs, we analyzed a set of genes that mark stemness. All four cell lines have increased mRNA levels of several cell surface markers commonly used for the identification of CSC subpopulation (ALDH1, CD133, CD25, LGR5, and MSI2) as well as genes that mediate cellular dedifferentiation (Twist, SNAIL, and VAX1) and the maintenance of the stem-cell phenotype (OCT4, MS1, SOX2, ZEB1; Supplementary Fig. S4C). Esophageal adenocarcinoma cell spheres

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Figure 3. Notch activity promotes the proliferation and/or survival of esophageal adenocarcinoma cell lines in vitro. A, immunofluorescence (IFC) staining for NICD (red) in OE33 cells treated by DAPT for 3 days. B, luciferase assays were performed in OE33 cells previously infected with 8XCSL reporter and subsequently treated by DAPT for 3 days. Error bars, SEM, *P = 0.0147; **P = 0.0164; ***P = 0.0004. C, mRNA levels of HES1 were determined by qPCR in OE33 cells treated by DAPT for 3 days. **P = 0.0085; ***P = 0.0022. D, cell viability assays were performed in OE33 and OE19 cells treated by DAPT for 4 days in low serum medium (1% FBS). **P < 0.05; ***P < 0.01; ****P < 0.001. E, colony formation assays were performed in OE33, JH-EsoAd1, and Het-1A cells treated by DAPT for 7 days. F, CSL knockdowns were verified by Western blotting and qPCR in OE33 cells. **P = 0.0016; ***P = 0.0020. G, cell viability assays done in OE33 cells infected with either control shRNA or shRNA against CSL. **P = 0.0011; ***P = 0.0022. H, colony formation assays were done in OE33 cells infected with shRNA against CSL and were quantified. **P = 0.0025. See also Supplementary Fig. S3.
were also much more efficient in forming tumors. Injecting $5 \times 10^5$ OE33 sphere cells resulted in tumors of equal or greater size than those obtained by injecting $5 \times 10^6$ parental cells, suggesting that the sphere cells are at least 10 times more efficient in forming xenograft tumors (Supplementary Fig. S4D). Furthermore, as few as $5 \times 10^4$ sphere-derived cells were able to initiate tumor formation in 5 weeks.

CSCs marker genes are sensitive to inhibition of Notch signaling

Once we established that esophageal adenocarcinoma cell spheres formed xenografts more efficiently, we tested whether inhibition of Notch would inhibit the growth of spheres. When compared with attached cells, spheres had a higher transcription of Notch target genes (Fig. 6B). Treatment of OE33 spheres with DAPT reduced the amount of NICD and Notch target gene transcription (Fig. 6B and C). GSI treatment also inhibits the ability of cell spheres to form secondary spheres (Fig. 6D). In contrast, FLO1/NICD cells containing a constitutive active form of Notch had a greater sphere-forming potential as compared with FLO1/Control cells (Fig. 6F).

To further confirm the role of Notch in the formation of esophageal adenocarcinoma spheres, we treated sphere cultures with GSI and measured the expression level of several stem-cell markers. The transcription of all stem-cell marker genes assayed was higher in cell spheres versus attached cells. Inhibition of the Notch pathway caused a significant decrease in transcription (P < 0.001) of several stem-cell marker genes, including ALDH, CD24, LGR5, SOX2, and TWIST1 (Fig. 6E). This indicates that this set of CSC marker genes is directly regulated by Notch. The transcription of another subset of genes tested was increased in sphere conditions but not significantly altered by GSI treatment (SNAIL, MSI2, NANOG, OCT4, and ZEB1), indicating that the increase in transcription is due to the loss of stemness but independent of Notch signaling in esophageal adenocarcinoma. GSI treatment of cell spheres increased the transcription of KLF4, which is in agreement with published data on the regulation of this gene by Notch. To validate this...
observation, we used FLO1/NICD cells and tested for the expression of two stem-cell marker genes, SOX2 and OCT4. Exogenous expression of NICD increased the transcription of SOX2 7 fold compared with control cells but did not alter the transcription of OCT4, indicating that SOX2 is specifically regulated by Notch (Fig. 6G). This was further confirmed by chromatin immunoprecipitation (ChIP), which demonstrates that NOTCH1 binds to the SOX2 promoter in both FLO1 and OE33 cells (Fig. 6H).

Esophageal adenocarcinoma CSC population is more sensitive to inhibition of Notch than bulk tumor cells

Because esophageal adenocarcinoma cell spheres show traits attributed to CSCs, we sought to determine if their maintenance was dependent on Notch signaling. We tested adherent cells separated according to their expression of aldehyde dehydrogenase (ALDH), a common CSC marker (21), for their ability to grow in sphere media. Analysis of four esophageal adenocarcinoma cell lines demonstrated that the ALDH-positive cells displayed a 2 to 5-times greater ability to form spheres when compared with ALDH-negative cells (Fig. 7A). In addition, treatment of attached cells with GSI decreased the ALDH$^+$ fraction by 40% to 60% (Fig. 7B). Therefore, the esophageal adenocarcinoma CSC population can be identified according to ALDH positivity and that this population of cells is more sensitive to inhibition of the Notch signaling than ALDH-negative cells.

Inhibition of Notch signaling sensitizes esophageal adenocarcinoma for treatment with 5-fluorouracil

The effect of blocking Notch on esophageal adenocarcinoma CSCs has been characterized by various CSC assays. Notch signaling seems to promote the growth maintenance of tumor-forming cells in esophageal adenocarcinoma. There is also clear evidence that higher level of Notch signaling in chemo-naïve patients is a marker for poor prognosis following
chemotherapy. To explore the role of Notch signaling on esophageal adenocarcinoma chemoresistance, we performed a survival assay using 5-fluorouracil (5-FU), a common agent used in esophageal adenocarcinoma chemotherapy. We chose to use OE33 and FLO1 cells for this experiment because they have very high and low levels of NICD, respectively (Fig. 6A). OE33 cells are insensitive to 5-FU treatment. Combination treatment of OE33 cells with GSI and 5-FU was more effective in killing OE33 (Fig. 7C). In contrast, FLO1 cells are moderately sensitive to 5-FU. When NICD is ectopically introduced to FLO1 cells, these cells (FLO1/NICD) now exhibit a resistance to 5-FU (Fig. 7D). Taken together, these results suggest that Notch signaling confers chemoresistance in esophageal adenocarcinoma cells. Therefore, it is establishing proof-of-concept that combinational therapy with GSI and other chemotherapeutic agents will be a useful strategy in esophageal adenocarcinoma treatment.

Discussion

The incidence of esophageal adenocarcinoma has shown a dramatic increase in the Western population (3, 22). The 5-year survival rate of esophageal adenocarcinoma is far below all other tumor types (5). Factors that have contributed to this poor 5-year survival are late diagnosis, resistance to chemotherapy, and metastatic disease (5, 6). Current treatment regimens for esophageal adenocarcinoma include NAC followed by surgical resection. Various chemotherapy regimens have been used and typically include 5-FU, oxiplatin, and docetaxel (18). In addition, radiotherapy can be given concomitantly with neoadjuvant chemotherapy. Despite these
treatment regimens, the clinical response is relatively poor and this leads to dismal 5-year outcome. Local response rates to these therapies vary but for the vast majority, treatment results in only a modest downsizing or no response at all. Less than 20% of patients display a complete pathologic response. However, it is these patients who obtain the greatest improvement in 5-year survival (18). Therefore, for improved clinical management of esophageal adenocarcinoma, a treatment must improve the local response to neoadjuvant therapy. Herein, we demonstrate that Notch signaling is critical for esophageal adenocarcinoma and underlies resistance to chemotherapy. We present evidence that Notch signaling is greater in the less differentiated tumors and drives a CSC phenotype. We demonstrate that Notch signaling is critical for these esophageal adenocarcinoma CSCs and that Notch regulates genes that establish stemness. Using PDX models, we clearly demonstrate that inhibition of Notch signaling by GSIs is efficacious in downsizing tumor growth. Moreover, we provide evidence that demonstrates Notch activity in a patient’s EUS-derived biopsy sample might predict outcome to chemotherapy. Taken together, our data strongly suggest that Notch signaling drives a significant proportion of esophageal adenocarcinomas. Notch signaling does so by establishing and maintaining a CSC-like population of cells, which also underlies resistance to chemotherapy. Therefore, inhibition of Notch depletes the CSC population and sensitizes cells to chemotherapeutic agents, which should lead to a better and more durable response to NAC. This study, therefore, provides a strong foundation for examining the efficacy of inhibitors of Notch signaling, such as GSI compounds, for the treatment of esophageal adenocarcinoma. Given that Notch seems to drive CSCs and resistance to chemotherapy, one might expect that the outcome of such clinical trials would be more profound on 5-year survival rate than in downsizing of the primary tumor itself as a single agent. Therefore, it would be a useful strategy to use GSIs to sensitize tumors to NAC and, therefore, improve both the local response and outcome of treatment. The use of a targeted therapy in combination with a typical chemotherapeutic and surgical treatment strategy will ultimately provide a more durable cure to this disease.

Figure 7. Notch inhibition selectively abolishes the CSC population, as well as sensitizes esophageal adenocarcinoma for 5-FU. A, tumor sphere assays were done in ALDH-sorted esophageal adenocarcinoma cell lines. B, flow cytometry analysis using ALDH in DAPT-pretreated esophageal adenocarcinomas. C, cell viability assays were performed in OE33 cells cotreated with 5-FU and DAPT for 4 days. **, \( P < 0.01 \); ***, \( P < 0.001 \). D, cell viability assay in FLO1/Control and FLO1/NICD cells treated with 5-FU for 4 days. ***, \( P < 0.001 \).
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank the members of the Capobianco laboratory for support and technical assistance. They also thank Drs. David G. Beer (Department of Surgery, Section of General Thoracic Surgery, University of Michigan Medical School, Ann Arbor, MI), Steven J. Hughes (Department of Surgery, University of Pittsburgh, Pittsburgh, PA), and James R. Eshleman and Anirban Maitra (Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD) for the esophageal adenocarcinoma cell lines.

Grant Support

This work was funded, in part, by grants from the National Cancer Institute (NCI R01CA083736-12AI, NCI R01CA125044-02, and NCI R01CA169805-01), and by funding from the Dewitt Daughtry Family Department of Surgery, and the Sylvester Comprehensive Cancer Center.

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Received July 11, 2014; accepted August 1, 2014; published OnlineFirst August 27, 2014.

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