Notch pathway activity identifies cells with cancer stem cell-like properties and correlates with worse survival in lung adenocarcinoma.


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Translational Relevance

Lung cancer is the leading cause of cancer-related death in the world, with no significant change in survival rates over the past 30 years. Cancer stem cells have the ability to self-renew and are resistant to chemotherapy treatment. Identifying pathways that regulate these cells may provide a valuable target for novel cancer treatment. In this manuscript, we identify Notch activity as a marker for cells with stem cell-like properties which could be targeted by specific drugs. We further show that Notch activity correlates with poor overall survival in lung cancer patients. This work suggests that using Notch inhibitors in the treatment of lung cancer has the potential to improve disease outcome.
Abstract

**Purpose:** The cancer stem cell theory postulates that tumors contain a subset of cells with stem cell properties of self-renewal, differentiation and tumor-initiation. The purpose of this study is to determine the role of Notch activity in identifying lung cancer stem cells.

**Experimental Design:** We investigated the role of Notch activity in lung adenocarcinoma utilizing a Notch GFP-reporter construct and a gamma-secretase inhibitor (GSI), which inhibits Notch pathway activity.

**Results:** Transduction of lung cancer cells with Notch GFP-reporter construct identified a subset of cells with high Notch activity (GFP-bright). GFP-bright cells had the ability to form more tumor spheres in serum-free media, and were able to generate both GFP-bright and GFP-dim (lower Notch activity) cell populations. GFP-bright cells were resistant to chemotherapy and were tumorigenic in serial xenotransplantation assays. Tumor xenografts of mice treated with GSI had decreased expression of downstream effectors of Notch pathway and failed to regenerate tumors upon reimplantation in NOD/SCID mice. Using multivariate analysis, we detected a statistically significant correlation between poor clinical outcome and Notch activity (reflected in increased Notch ligand expression or decreased expression of the negative modulators), in a group of 441 lung adenocarcinoma patients. This correlation was further confirmed in an independent group of 89 adenocarcinoma patients where Hes-1 overexpression correlated with poor overall survival.

**Conclusions:** Notch activity can identify lung cancer stem cell-like population and its inhibition may be an appropriate target for treating lung adenocarcinoma.
Introduction

Lung cancer is the leading cause of cancer-related death in the world. In the United States, 221,000 new cases of lung cancer and 157,000 lung cancer deaths were estimated in 2011 (1). Over the past three decades, there has been little change in the fifteen percent five-year survival rate (2). For patients with advanced-stage disease, current treatment with chemotherapy and radiotherapy is rarely curative. Thus, novel approaches to target tumor cells responsible for resistance and recurrence are needed.

The concept of a rare subpopulation of cancer stem cells (CSCs) responsible for tumor initiation and maintenance was suggested more than 50 years ago (3). In 1994, John Dick completed seminal work to substantiate the existence of such cells in hematological malignancies. His laboratory identified a rare cell population in human acute myeloid leukemia (AML) that has the capacity to initiate AML after transplantation into NOD/SCID mice (4). Almost a decade later, CSCs were identified in human breast cancer with the capacity for self-renewal and recapitulation of the primary tumor in NOD/SCID recipients (5). Since then, CSCs have been documented in other solid tumors, including brain, prostate, colon, pancreatic and lung cancer (6-11). Ample data suggest that CSCs are relatively quiescent and are resistant to chemotherapy and radiation (12-14). In fact, exposing cell lines to multiple cytotoxic agents selects for surviving cells with CSC properties (15). Several pathways such as Notch and Hedgehog appear to play an essential role in CSC maintenance (16-19).

The evolutionarily conserved Notch signaling pathway affects development in organisms ranging from sea urchins to humans (20) and plays a crucial role in stem cell control and cell-fate determination (21). In mammals, four distinct Notch receptors

4
interact with various ligands that initiate a series of proteolytic steps leading to the release of the Notch intracellular domain (NICD) (22-24). Gamma secretase is the enzyme responsible for the final cleavage reaction leading to the release of the NICD. NICD then translocates to the nucleus and activates target-gene transcription.

The role of Notch signaling in lung cancer was first suggested when overexpression of Notch was detected in non-small cell lung cancer (NSCLC) cell lines (25). Furthermore, Notch3 was found to be expressed in 39% of resected human lung cancers and its inhibition reduced growth in soft agar (26). Notch signaling inhibition with a gamma secretase inhibitor reduced tumor cell proliferation, induced apoptosis, and inhibited \textit{in vivo} growth (27). In addition, hypoxia increased Notch signaling and sensitized lung tumor cell lines to inhibition by gamma secretase inhibitors (28). Moreover, Notch activating mutation has been detected in 10% of NSCLC, the first evidence of Notch mutation in solid tumors (29). Although gamma secretase inhibitors (GSIs) have been shown to induce apoptosis in various types of cancer cell lines and to inhibit angiogenesis and tumor growth \textit{in vivo} (30-32), the effect of Notch inhibition on CSCs has not been explored.

In a previous report, we found that an embryonic stem cell–like signature identified poorly differentiated lung adenocarcinoma patients and was associated with worse overall survival in lung adenocarcinoma (33). Further analysis showed that the Notch pathway correlated with this embryonic stem cell–like signature (unpublished data). In this manuscript, we provide direct evidence that Notch activity selects for lung cancer cells with CSC properties. Inhibiting the Notch pathway prevents sphere formation and \textit{in vivo} tumor reimplantation. Furthermore, we show that Notch activity is associated with
worse outcome in patients with lung adenocarcinoma, suggesting a potential role for inhibiting Notch activity as a new therapeutic approach for these patients.

**Materials and Methods**

**Lung Cancer Cell Lines, Compounds, Primary Lung Adenocarcinoma Specimens and Gene Sets**

NSCLC cell lines NCI-H1299, NCI-H358, NCI-H441, NCI-H460, and A549 were obtained from American Type Culture Collection (ATCC), maintained in RPMI1640 with 10% FBS and used in all experiments at 70% to 80% confluency. All the cell lines were used within six months of resuscitation and have been authenticated by ATCC with identifiable short tandem repeat (STR) loci. The γ-secretase inhibitor MRK-003 was provided by Merck & Co., Inc. and stocks at 10 mM in DMSO were used in this study. Docetaxel was purchased from Sanofi-Aventis and cisplatin was purchased from Sigma-Aldrich, Inc. The complete data for the human primary adenocarcinomas used in this study are available in Hassan *et al.* (33). A summary of the clinical variables is provided in Supplementary Table 1. The gene-sets list of Notch pathway categorized by functionality is shown in Supplementary Table 2 (34). Microarray gene expression data on 443 human lung adenocarcinomas were downloaded from NCI caARRAy as presented in original manuscript (35). Raw data were processed by log 2 transformation of the expression values, and the mean center expression level for each gene across all samples was determined. The expression was represented relative to the mean of each gene. The processed expression data are provided as Supplementary Table S3.
Plasmid and stable transfectants

A pGreenFire1-Notch plasmid that expressed destabilized copGFP reporter and firefly luciferase under the control of four Notch response elements and a minimal CMV promoter was obtained from System Biosciences. NSCLC cell lines were used to establish the stable transfectants by using FuGene 6 tranfection reagent (Roche Diagnostics).

Quantitative real-time reverse transcription-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen), and the SYBRgreen quantitative real-time reverse transcription-PCR (Q-PCR) was carried out in an ABI PRISM 7900HT Sequence Detection System with the Taqman Universal PCR Master Mix (Applied Biosystems). The primer sets for all the genes was purchased from Applied Biosystems and the list is provided in supplementary material. The expression levels of the transcripts were calculated using the linear exponential phase of amplification throughout 10 to 40 cycles and β-actin was used as the transcript internal control for normalization of each reaction.

MTT assay, cell cycle analysis, and apoptosis assay with flow cytometry

Transfected cells were plated at a density of 5,000 or 2,500 cells per well when using a 96-well plate to perform the standard MTT proliferation assay of 24 hours or 48 hours. Each experiment was repeated at least thrice with similar results. Apoptosis was measured following MRK-003 treatment by using APC-Annexin V from BD Biosciences with a MoFlo XDP flow cytometer (Beckman Coulter, Inc.).

In vivo tumorigenicity and MRK-003 treatments

Athymic four to six week-old Nod/Scid mice were used. The xenograft mice were generated with lung cancer cell line NCI-H1299 and the stable transfectants H1299-
Notch-GFP by subcutaneous injections in the flank area. For reimplantation studies, tumors were removed, chopped and processed with collagenase for one to two hours at 37°C. Cells were then washed with PBS, trypsinized, and passed through a 40 micron filter. The single cells obtained were labeled with H-2Kd antibody and DAPI and then sorted with flow cytometry. Alive human cells were reinoculated subcutaneously to determine reimplantation capacity. For in vivo treatment studies, mice were randomly assigned to different groups when the tumors were palpable. Tumors were measured weekly using a caliper and tumor size was calculated using the following formula: Tumor Volume = Length x Width²/2.

**Sphere formation assay and Generation of tumor xenograft from single cell**

Single GFP-bright and GFP-dim cells were sorted by flow cytometry and directly plated at one cell per well into a 96-well Ultra Low Cluster plate (Corning Inc.). Cells were then allowed to grow for twelve to fourteen days in the MammoCult Basal Medium (StemCell Technologies), supplemented with 20ug/ml Hydrocortisone and 10nM Heparin. The number of wells containing spherical organoids was counted. After dissociation, the cells from spherical organoids were serially diluted and plated into another 96-well Ultra Low Cluster plate for measuring the secondary sphere formation.

**Statistical analysis**

The xenograft tumors of treated groups and control groups were compared at different time points. Unless specifically stated, statistical analysis in comparative experiments both in vivo and in vitro was performed with the unpaired two-sided Student’s t-test. A twofold change in the expression levels of Notch pathway functional molecules was considered significant. P values were calculated using the log-rank test. Differences were considered significant when the P value was <0.05. Kaplan-Meier
survival curve was used when comparing the group of adenocarcinoma patients with higher Notch pathway activity to all other individuals.

**Results**

**Establishing stable NSCLC cell lines with Notch reporter activity**

We used a lentiviral Notch reporter vector that drives the bicistronic expression of GFP and Luciferase under the minimal essential CMV promoter downstream of Notch transcriptional response elements. Using this construct, we transfected multiple NSCLC cell lines and detected GFP expression in 0.5-2% of the population by flow cytometry (Supplementary Fig. S1A, B). We established stable cell lines expressing the Notch reporter through sorting GFP-bright cells by flowcytometry and expanding the GFP-bright and GFP-dim cells in culture for multiple passages (Supplementary Fig. S1C-H, Supplementary Fig. S2A-H). The expression of Notch pathway components varied between the GFP-bright and GFP-dim cells. The expression of Notch-2 and Notch-4 receptors was higher in the GFP-bright population while Notch-1 and Notch-3 receptors were more expressed in GFP-dim population (Supplementary Fig. S3A). The jagged family expression profile showed enrichment in the GFP-dim population, suggesting a potential paracrine effect on GFP-bright cells (Supplementary Fig. S3B).

To determine that GFP-bright cells have active Notch pathway, we assessed the expression of Hes1 and Hey1, the main downstream effectors of Notch pathway. We found an increased expression of Hes1 and Hey1 in NCI- H1299 GFP-bright population as compared to GFP-dim population (Supplementary Fig. S4A). Furthermore, we used soluble DLL4 as a competitive inhibitor of Notch pathway and detected a decrease in the luciferase activity in the GFP-bright population (Supplementary Fig. S4B).
Treating NCI-H1299 GFP-bright cells with GSI inhibited the ability of these cells to form tumors (Supplementary Fig. S4C). Furthermore, NCI-H1299 cells transduced with constitutively active NICD1 (Notch Intracellular Domain 1) and NICD2 rescued these cells from the GSI effect on sphere formation, while NICD3 did not. These results provide further evidence that GSI inhibits the Notch pathway and that specific Notch receptors are involved in sphere formation. Furthermore, we assessed NICD2 expression, by western blotting, in GFP-bright cells with and without GSI treatment. GSI treated cells had decrease in NICD1 expression, in a dose dependent manner, as compared to untreated cells. (Supplementary Fig. S4D) To ensure that the reporter vector was integrated in the GFP-dim cells genome, we performed PCR detecting GFP in the GFP-dim cells (Supplementary Fig. S4E). This indicates the presence of the vector in GFP-dim cells, but a lack of Notch activity, since GFP is not expressed.

**Notch activity identifies cells with stem-cell like properties**

We observed a higher growth rate in the GFP-dim cells when compared to GFP-bright cells. However, GFP-bright cells were able to generate both GFP-bright and -dim populations, while GFP-dim cells failed to generate GFP-bright cells (Supplementary Fig. S1C-H). To determine whether Notch activity is required for self-renewal in vitro, we performed sphere forming assay. GFP-bright cells formed more primary and secondary spheres than GFP-dim cells (15%, 46% vs. 2%, 1% respectively) suggesting a role for Notch signaling in sphere formation and self-renewal (Fig. 1). This was confirmed in other cell lines as shown in Supplementary Fig. S4F.

In order to determine the ability of GFP-bright and GFP-dim cells to initiate tumor xenografts in mice, we subcutaneously implanted 10,000, 1000 and 100 cells from...
each cell population in NOD/SCID mice. Both GFP-bright and GFP-dim cells at 10K and 1K were able to form primary xenografts (Fig. 2A), while 100 cells did not (Table 1). When both tumors were evaluated by flowcytometry, GFP-bright cells generated both GFP-bright and -dim populations while GFP-dim tumors had a negligible GFP-bright population (data not shown). This suggests that GFP-bright cells not only initiate tumors but also generate heterogeneous cell population. When secondary implantation was attempted, GFP-dim cells failed to generate tumors after six month of inoculation, while GFP-bright readily formed secondary tumors in four to six weeks (Table 1). One hundred GFP-bright cells were sufficient to successfully generate xenograft tumors in NOD/SCID mice (Fig. 2). Serial reimplantation of GFP-bright xenograft tumors continued to be successful for four generations.

**Single Notch active Cell Generates xenograft in mouse model**

To determine whether a single GFP-bright cell is capable of generating tumors, we sorted single GFP-bright and GFP-dim cells into each well of 96 well plates to grow in serum-free media. After one week, multiple NOD/SCID mice were injected subcutaneously, each with a sphere formed from a single cell. After ten weeks, GFP-bright spheres formed xenografts consisting of both GFP-bright and GFP-dim cell populations, while GFP-dim cells failed to generate tumors. This provides further evidence that GFP-bright cells contain a population capable of self-renewal, proliferation and differentiation. Xenograft tumors from parental H1299, primary GFP-bright xenograft, secondary GFP-bright xenograft, and single cell sphere xenograft tumors were similar histologically (Fig. 2 E-H).
Furthermore, we assessed whether GFP-bright cells presence would impact the ability of GFP-dim cells to form tumors. We transduced GFP-dim cells with constitutively active Ds-red vector and selected these cells by flow cytometry. We then mixed and subcutaneously inoculated equal numbers of GFP-dim-Ds-red-pos cells with GFP-bright cells in NOD/SCID mice. After xenograft formation, tumor tissues were assessed for Ds-red and GFP expression by flow cytometry. There were no detectable Ds-red cells and more importantly, there was no evidence of cells simultaneously expressing GFP and Ds-red. This confirms that GFP-dim cells lack the capability to initiate tumors even in the presence of GFP-bright cells.

**Notch active cells are resistant to chemotherapy.**

Resistance to chemotherapy has been identified as a feature of cancer stem cells and was used to select for cancer stem cells (15). To evaluate whether Notch activity correlates with resistance to chemotherapy, we treated GFP-bright and GFP-dim cells with cisplatin or docetaxel. Cisplatin is usually used in first-line treatment and docetaxel is used in second- or third-line treatment of lung cancer. MTT assays showed decreased viability of GFP-dim cells upon treatment with chemotherapy agents as compared to GFP-bright cells (data not shown). To determine whether the decrease in viability was due to apoptosis induced by chemotherapy agents, we measured Annexin V expression with flow cytometry. H1299 GFP-bright cells showed sixfold less Annexin V staining as compared to GFP-dim cells, when treated with both agents (Fig. 3A-J). This was confirmed in other cell lines, H358 (Fig. 3K), H460 (Fig. 3L), A549 (Fig. 3M), and H441 (Fig. 3N), indicating that GFP-bright cells are significantly more resistant to chemotherapy as compared to GFP-dim cells and are enriched after chemotherapy exposure.
**GSI effect on lung cancer xenografts in vivo**

Xenograft models utilizing NOD/SCID mice with subcutaneously injected NCI-H1299 cells were used to evaluate the in vivo effects of MRK003 on non-small cell lung cancer lines. Mice were divided into four groups that were respectively treated with MRK003 alone, docetaxel alone, MRK003 plus docetaxel, and no treatment. Treatment was initiated when tumor volume reached five cubic millimeters.

Single-agent MRK003 resulted in modest non-statistically significant growth inhibition when compared to the control group. Docetaxel alone (p= 0.003) or in combination with MRK003 (p= 0.001) caused significant inhibition of tumor growth (Supplementary Fig. S5A). Exposure to MRK003 decreased the expression of downstream effectors of Notch pathway confirming that MRK003 targets Notch pathway activity (Supplementary Fig. S5B). Interestingly, tumors that were removed from MRK003-treated NCI-H1299 xenograft mice and reimplanted into naive NOD/SCID mice failed to regenerate tumors (0/6), while those removed from mice treated with docetaxel alone did generate new tumors (3/3) upon reimplantation.

**Notch pathway activity correlates with poor overall survival**

Notch 1 activity has been correlated with poor prognosis in lung cancer (18). To determine the role of the Notch pathway in the outcomes of patients with lung adenocarcinoma (Table S1), we evaluated the correlation between overall survival and the expression of Notch ligands, receptors, downstream effectors and inhibitors by multivariate analysis. We found that increased Notch ligand expression (p= 0.0423) and decreased Notch pathway inhibitor expression (p=0.0002) correlated with poor prognosis (Table 2). Kaplan-Meier analyses of overall survival showed that patients carrying
tumors with decreased expression of Notch inhibitors had a significantly worse five-year overall survival than patients with tumors that had increased expression (Fig. 4A).

To further validate these findings, we used an independent cohort of 89 primary human lung adenocarcinoma. Immunohistochemical staining for Hes-1 protein was performed on the tissue microarray and the level of expression analyzed (Supplementary Fig. S6). Indeed, Kaplan-Meier analysis of overall survival showed that patients whose tumors overexpress Hes-1 had a significantly worse five-year overall survival (p=0.038) than patients with tumors that had low expression (Figure 4B).

These data suggest that Notch pathway activation leads to worse clinical outcomes. Therefore, interventions resulting in overall Notch inhibition might be a promising therapeutic strategy to improve survival in lung cancer patients.

Discussion

Serial analysis of gene expression (SAGE) profiling of non-small cell lung cancer specimens shows high expression of downstream effectors of Notch, suggesting an important role for Notch signaling in lung carcinogenesis (36). In addition, Notch1 activation mutations have been detected in 10% NSCLC primary tumors (29); however, we only detected synonymous SNPs in the cell lines used in this manuscript (Supplementary Fig. S7). Moreover, other studies showed that GSI inhibits the Notch pathway leading to reduced proliferation, apoptosis and in vivo growth inhibition (27). However, the role of Notch pathway and the effect of its inhibition by GSI in lung CSCs, has not yet been thoroughly probed. A recent publication suggests that Notch inhibition decreases the number of aldehyde dehydrogenase (ALDH) positive cells that have the ability to self-renew (37).
To provide further evidence of the potential role of Notch activity in cancer stem cells, we used a Notch GFP-reporter vector that is activated when NICD translocates to the nucleus. These studies show that only the subpopulation which possesses Notch activity has the ability to form spheres and is resistant to chemotherapy \textit{in vitro}. This correlation between Notch activity and sphere formation has also been reported in breast and brain tumors. The presence of activating Notch gene rearrangement in primary tumors or overexpressing constitutively active NICD correlated with mammosphere forming ability (38, 39). Similarly, overexpression of NICD1 in glioblastoma cell lines increased neurosphere formation capability (40). Furthermore, cells with high Notch activity had increased expression of known stem cell related genes (Supplementary Fig. S8). \textit{In vivo}, only cells that had an active Notch pathway were able to self-renew. Although multiple reports have identified various markers that can select for a putative lung cancer stem cell subpopulation, it has been challenging to reproduce these findings. CD133 was first reported by Eramo \textit{et. al.} to be a selective marker for lung CSCs and soon after ALDH activity was identified as a marker for cells with stem cell properties (10, 11). In our hands, CD133 and ALDH activity failed to select a population of cells with robust cancer stem cell features. This raises concerns regarding the validity of specific stem cell markers across various lung cancer model systems. The potential importance of these markers lies in identifying specific populations of cells that possess the unique capacity for self-renewal and resistance to treatment. Acknowledging the limitations of such markers, one should not dismiss the value of recognizing critical self-renewal pathways that if targeted might have a significant impact on disease progression, recurrence and ultimately patient survival.
Our analysis of Notch activity in primary tumors showed a strong correlation between Notch activity, either through increased expression of ligands or through decreased expression of inhibitors, and poor clinical outcome. Although the expression of downstream genes did not correlate with outcome at the RNA level, Hes-1 protein expression did correlate with poor overall survival. This could be due to post translational modifications as well as to crosstalk with other pathways, such as Wnt or Hedgehog (41, 42). However, our findings are in line with data published by Westhoff et al. indicating that Notch 1 activity as determined by immunohistochemistry correlates with poor outcome in lung primary NSCLCs.

In this manuscript, we delineated the role of Notch activity in lung cancer stem cells and its correlation with clinical outcome. This provides substantiation for the crucial role of Notch activity in primary lung tumors and supports the concept that inhibition of the Notch pathway, which targets a subpopulation of cells with self-renewal capacity, is a rational strategy for the treatment of lung cancer.

Acknowledgments

We would like to recognize our colleagues, Dr. Suling Liu and Dr. Christophe Ginestier, for helpful discussions throughout this project. We appreciate Samantha Hodge-Williams assistance in editing this manuscript. We thank Merck & Co., Inc. for providing the GSI (MRK003).

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References


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Tables

**Table 1:** NOD/SCID mice subcutaneously injected with GFP-bright and -dim cells initiated primary xenograft with 10K and 1K cells, but failed to initiate any xenograft with 100 cells. Notch reporter positive cells derived from primary xenografts successfully generate secondary xenografts with as low as 100 cells. Notch negative cells failed to regenerate secondary xenografts with 10K cells.

<table>
<thead>
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<th>Primary Xenograft</th>
<th>10K</th>
<th>1K</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-bright</td>
<td>3/4</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td>GFP-dim</td>
<td>3/4</td>
<td>3/4</td>
<td>0/4</td>
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<table>
<thead>
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<th>Secondary Xenograft</th>
<th>10K</th>
<th>1K</th>
<th>100</th>
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<tr>
<td>GFP-bright</td>
<td>6/6</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>GFP-dim</td>
<td>0/6</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>
**Table 2:** Cox Multivariate and univariate analysis of overall survival in human lung adenocarcinoma based on Notch pathway category expression.

<table>
<thead>
<tr>
<th>Notch pathway Category</th>
<th>Multivariate* p value</th>
<th>beta</th>
<th>Univariate p value</th>
<th>beta</th>
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<tbody>
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<td>Ligands</td>
<td>0.0423</td>
<td>0.15</td>
<td>0.1005</td>
<td>0.12</td>
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<tr>
<td>Downstream effectors</td>
<td>0.8329</td>
<td>0.02</td>
<td>0.8218</td>
<td>0.02</td>
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<td>Receptors</td>
<td>0.1136</td>
<td>-0.12</td>
<td>0.2443</td>
<td>-0.08</td>
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<td>0.6712</td>
<td>-0.03</td>
<td>0.8505</td>
<td>0.01</td>
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<tr>
<td>Transcription factors</td>
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<td>-0.08</td>
<td>0.8117</td>
<td>-0.02</td>
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<td>0.3798</td>
<td>0.07</td>
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<tr>
<td>Modulators, negative</td>
<td><strong>0.0002</strong></td>
<td>-0.30</td>
<td><strong>0.0005</strong></td>
<td>-0.26</td>
</tr>
</tbody>
</table>

* Age, gender, stage, differentiation and each Notch pathway category were included in each multivariate Cox model analysis.
Figures

**Figure 1. GFP-dim and GFP-bright sphere formation.** GFP-dim sphere: (A) bright field; (B) GFP filter; (C) merged. GFP-bright sphere: D) bright field, E) GFP filter, F) merged. G) GFP-dim secondary sphere, H) GFP-bright secondary sphere. (All 40X magnification); (I) Average primary and secondary sphere formation of GFP-bright and GFP-dim cells. GFP-bright cells form more primary (p=0.00048) as well as secondary (p=0.0008) spheres as compared to GFP-dim cells.

**Figure 2. GFP-bright and GFP-dim xenografts.** Bioluminescence imaging of GFP-bright and GFP-dim secondary xenografts, showing the presence of tumor only in animals injected with GFP-bright cells. (A) Back and (B) Front of NOD/SCID mice injected with 10,000, 1000 or 100 GFP-bright cells subcutaneously showing tumor mass and increased bioluminescence after luciferin injection. (C) Back and (D) Front of NOD/Scid mice injected with 10,000, 1000 or 100 GFP-dim cells subcutaneously with absence of tumor formation. Hematoxylin and eosin staining of xenograft tumors showing similar histology at 10X and 40X. (E) Parental H1299; (F) Primary GFP-bright xenograft; (G) Secondary GFP-bright xenograft; (H) Single cell sphere xenograft tumors.

**Figure 3. Annexin V flow cytometry apoptosis assay.** (A) Control, untreated NCI-H1299 GFP-bright and -dim cells; (B) 2% of untreated GFP-dim cells are AnnexinV-APC positive; (C) 1.7% of GFP-bright cells are Annexin V-APC positive; (D) Docetaxel treated GFP-bright and -dim cells; (E) 61% of Docetaxel treated GFP-dim cells are Annexin V-APC positive; (F) 10% of Docetaxel treated GFP-bright cells are Annexin V-APC positive; (G) Cisplatin treated GFP-bright and -dim cells; (H) 25% of Cisplatin...
treated GFP-dim cells are Annexin V- APC positive; (I) 7% of Cisplatin treated GFP-bright cells are Annexin V- APC positive. Annexin V apoptosis assay after exposure to Docetaxel and Cisplatin in different cell lines (average of three experiments); (J) NCI-H1299; (K) NCI-H358; (L) NCI-H460; (M) A549; (N) NCI-H441. Annexin V expression was significantly higher in all treated GFP-bright cells as compared to treated GFP-dim cells except the docetaxel treated NCI-H358 cells where the difference wasn’t statistically significant (p=0.38)

**Figure 4.** (A) Kaplan–Meier curve of overall survival for patients with high and moderate expression of Notch negative modulators compared to those with low expression. Hazard ratio 0.52 (95% CI, 0.39-0.70). (B) Kaplan–Meier curve of overall survival for patients with high and moderate expression of Hes-1 compared to those with low expression. Hazard ratio HR 2.44 (95% CI, 1.02 – 5.81).
Figure 2
Figure 3

A) Control

D) Docetaxel

G) Cisplatin

B) GFP-dim

C) GFP-bright

E) GFP-dim

F) GFP-bright

H) GFP-dim

I) GFP-bright

2%

1.7%

61%

10%

25%

7%
Figure 4

A

B

Survival Probability

Survival Probability

Survival Time (m)

Survival Time (m)

P = 0.000007

P = 0.038

Low 1/3

Medium-High 2/3

Low 1/3

Medium-High 2/3
Clinical Cancer Research

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