

Notch Pathway Activity Identifies Cells with Cancer Stem Cell-like Properties and Correlates with Worse Survival in Lung Adenocarcinoma

Khaled A. Hassan¹, Luo Wang¹, Hasan Korkaya¹, Guoan Chen², Ivan Maillard¹, David G. Beer², Gregory P. Kalemkerian¹, and Max S. Wicha¹

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 using a Notch GFP reporter construct and a γ -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 443 patients with lung adenocarcinoma. This correlation was further confirmed in an independent group of 89 patients with adenocarcinoma in which 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. *Clin Cancer Res*; 19(8); 1972–80. ©2013 AACR.

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 3 decades, there has been little change in the 15% 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 (CSC) 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, 4 distinct Notch receptors interact with various ligands that initiate a series of proteolytic steps leading to the release of the Notch intracellular domain (NICD; refs. 22–24). γ -Secretase is the enzyme responsible for the final cleavage reaction leading to

Authors' Affiliation: ¹Department of Internal Medicine and ²Department of Surgery, University of Michigan, Ann Arbor, Michigan

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Corresponding Author: Khaled A. Hassan, University of Michigan, 7310 Cancer Center, 1500 E. Medical Center Drive, Ann Arbor, MI 48109. Phone: 734-615-2939; Fax: 734-936-4940; E-mail: khaledh@med.umich.edu

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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 the article, we identify Notch activity as a marker for cells with stem cell–like properties that could be targeted by specific drugs. We further show that Notch activity correlates with poor overall survival in patients with lung cancer. This work suggests that using Notch inhibitors in the treatment of lung cancer has the potential to improve disease outcome.

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 γ -secretase inhibitor (GSI) reduced tumor cell proliferation, induced apoptosis, and inhibited *in vivo* growth (27). In addition, hypoxia increased Notch signaling and sensitized lung tumor cell lines to inhibition by GSIs (28). Moreover, Notch activating mutation has been detected in 10% of NSCLCs, the first evidence of Notch mutation in solid tumors (29). Although GSIs have been shown to induce apoptosis in various types of cancer cell lines and to inhibit angiogenesis and tumor growth *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 patients with lung adenocarcinoma 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 the article, we provide direct evidence that Notch activity selects for lung cancer cells with CSC properties. Inhibiting the Notch pathway prevents sphere formation and *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 RPMI-1640 with 10% FBS and used in all experiments at 70% to

80% confluency. All the cell lines were used within 6 months of resuscitation and have been authenticated by ATCC with identifiable short tandem repeat (STR) loci. The GSI MRK-003 was provided by Merck & Co., Inc., and stocks at 10 mmol/L in dimethyl sulfoxide 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 and colleagues (33). A summary of the clinical variables is provided in Supplementary Table S1. The gene set list of Notch pathway categorized by functionality is shown in Supplementary Table S2 (34). Microarray gene expression data on 443 human lung adenocarcinomas were downloaded from NCI caARRAy as presented in original article (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 4 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 transfection 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 (qPCR) 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 conduct the standard MTT proliferation assay of 24 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 4- to 6-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 1 to 2 hours at 37°C. Cells were then washed with PBS, trypsinized, and passed through a 40- μ m filter. The single cells obtained were labeled with H-2Kd antibody and 4',6-diamidino-2-phenylindole (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 \times 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 12 to 14 days in the MammoCult Basal Medium (StemCell Technologies), supplemented with 20 μ g/mL hydrocortisone and 10 nmol/L 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 conducted with the unpaired 2-sided Student *t* test. A 2-fold 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 *P* < 0.05. Kaplan–Meier survival curve was used when comparing the group of patients with adenocarcinoma 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% to 2% of the population by flow cytometry (Supplementary Fig. S1A and S1B). We established stable cell lines expressing the Notch reporter through sorting GFP-bright cells by flow cytometry and expanding the GFP-bright and GFP-dim cells in culture for multiple passages (Supplementary Figs. S1C–S1H and S2A–S2H). 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 whereas 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 Hes-1 and Hey1, the main downstream effectors of Notch pathway. We found an increased expression of Hes-1 and Hey1 in NCI-H1299 GFP-bright population as compared with 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 whereas 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 decreased NICD2 expression, in a dose-dependent manner, as compared with untreated cells (Supplementary Fig. S4D). To ensure that the reporter vector was integrated in the GFP-dim cells genome, we conducted 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, as GFP is not expressed.

Notch activity identifies cells with stem cell-like properties

We observed a higher growth rate in the GFP-dim cells than in GFP-bright cells. However, GFP-bright cells were able to generate both GFP-bright and -dim populations, whereas GFP-dim cells failed to generate GFP-bright cells (Supplementary Fig. S1C–S1H). To determine whether Notch activity is required for self-renewal *in vitro*, we conducted 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.

To determine the ability of GFP-bright and GFP-dim cells to initiate tumor xenografts in mice, we subcutaneously implanted 10,000, 1,000, and 100 cells from each cell population in NOD/SCID mice. Both GFP-bright and GFP-dim cells at 10,000 and 1,000 were able to form primary xenografts (Fig. 2A) whereas 100 cells did not (Table 1). When both tumors were evaluated by flow cytometry, GFP-bright cells generated both GFP-bright and -dim populations whereas 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

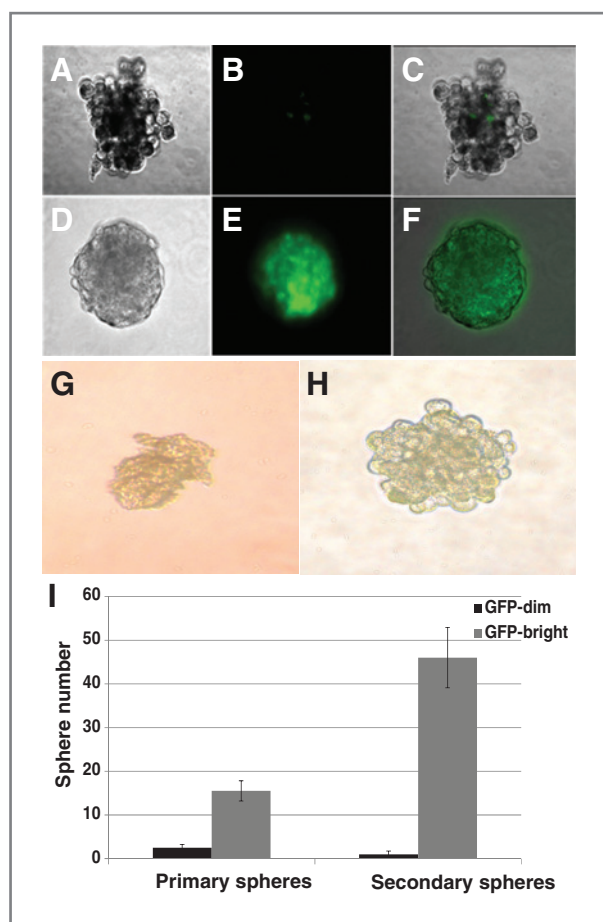


Figure 1. GFP-dim and GFP-bright sphere formation. GFP-dim sphere: A, bright field; B, GFP filter; and C, merged. GFP-bright sphere: D, bright field; E, GFP filter; and F, merged. G, GFP-dim secondary sphere. H, GFP-bright secondary sphere. (All $\times 40$ magnification). I, average primary and secondary 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 with GFP-dim cells.

secondary implantation was attempted, GFP-dim cells failed to generate tumors after 6 month of inoculation, whereas GFP-bright readily formed secondary tumors in 4 to 6 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 4 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 1 week, multiple NOD/SCID mice were injected subcutaneously, each with a sphere formed from a single cell. After 10 weeks, GFP-bright spheres formed xenografts consisting of both GFP-bright and GFP-dim cell populations, whereas 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. 2E–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 CSCs and was used to select for CSCs (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 with 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 6-fold less Annexin V staining than 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 than GFP-dim cells and are enriched after chemotherapy exposure.

GSI effect on lung cancer xenografts *in vivo*

Xenograft models using NOD/SCID mice with subcutaneously injected NCI-H1299 cells were used to evaluate the *in vivo* effects of MRK-003 on NSCLC cancer lines. Mice were divided into 4 groups that were respectively treated with MRK-003 alone, docetaxel alone, MRK-003 plus docetaxel, and no treatment. Treatment was initiated when tumor volume reached 5 mm^3 .

Single-agent MRK-003 resulted in modest nonstatistically significant growth inhibition when compared with the control group. Docetaxel alone ($P = 0.003$) or in combination with MRK-003 ($P = 0.001$) caused significant inhibition of tumor growth (Supplementary Fig. S5A). Exposure to MRK-003 decreased the expression of downstream effectors of Notch pathway confirming that

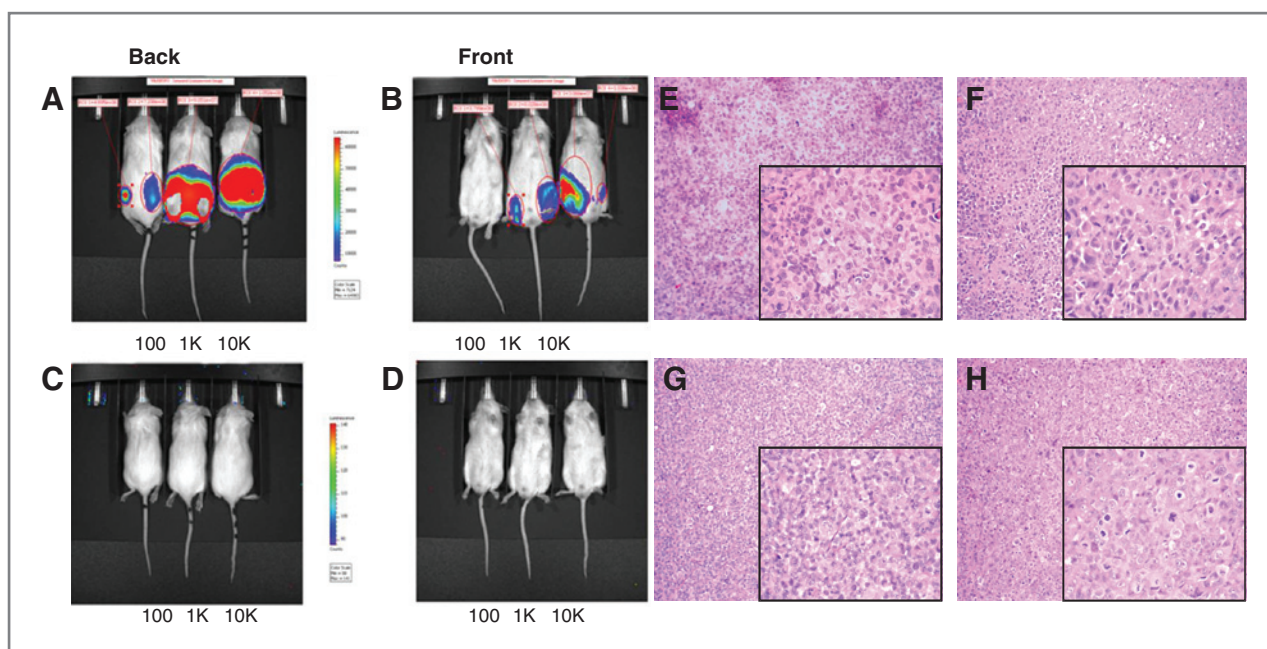


Figure 2. GFP-bright and GFP-dim xenografts. Bioluminescence imaging of GFP-bright and GFP-dim secondary xenografts, showing the presence of tumor only in animals injected with GFP-bright cells. Back (A) and front (B) of NOD/SCID mice injected with 10,000, 1,000, or 100 GFP-bright cells subcutaneously showing tumor mass and increased bioluminescence after luciferin injection. Back (C) and front (D) of NOD/SCID mice injected with 10,000, 1,000, or 100 GFP-dim cells subcutaneously with absence of tumor formation. Hematoxylin and eosin staining of xenograft tumors showing similar histology at $\times 10$ and $\times 40$. Parental H1299 (E), primary GFP-bright xenograft (F), secondary GFP-bright xenograft (G), and single-cell sphere xenograft tumors (H).

MRK-003 targets Notch pathway activity (Supplementary Fig. S5B). Interestingly, tumors that were removed from MRK-003-treated NCI-H1299 xenograft mice and reimplanted into naive NOD/SCID mice failed to regenerate tumors (0 of 6), whereas those removed from mice treated with docetaxel alone did generate new tumors (3 of 3) upon reimplantation.

Notch pathway activity correlates with poor overall survival

Notch1 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 (Supplementary 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 5-year overall survival than patients with tumors that had increased expression (Fig. 4A).

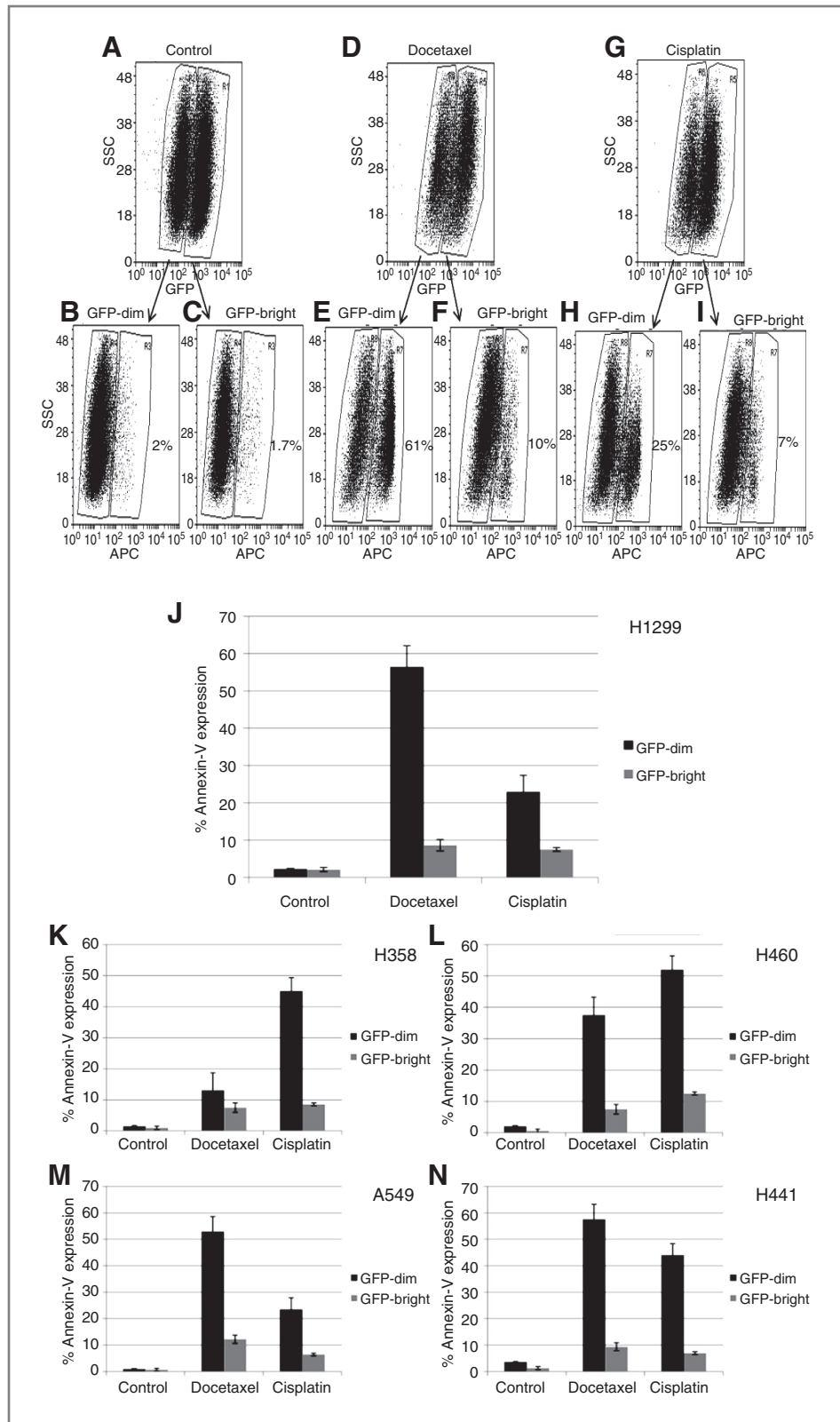
To further validate these findings, we used an independent cohort of 89 primary human lung adenocarcinomas. Immunohistochemical staining for Hes-1 protein was conducted on the tissue microarray and the level of expression analyzed (Supplementary Fig. S6). Indeed, Kaplan–Meier analysis of overall survival showed that patients whose

Table 1. Primary and secondary xenograft formation from GFP-bright and -dim cells in subcutaneously injected NOD/SCID mice

Primary xenograft	10,000	1,000	100
GFP-bright	3/4	3/4	0/4
GFP-dim	3/4	3/4	0/4
Secondary xenograft	10,000	1,000	100
GFP-bright	6/6	3/3	3/3
GFP-dim	0/6	0/3	0/3

NOTE: 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 10,000 cells.

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 Annexin V-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 expression was significantly higher in all treated GFP-bright cells than in treated GFP-dim cells except the docetaxel-treated NCI-H358 cells where the difference was not statistically significant ($P = 0.38$).



tumors overexpress Hes-1 had a significantly worse 5-year overall survival ($P = 0.038$) than patients with tumors that had low expression (Fig. 4B).

These data suggest that Notch pathway activation leads to worse clinical outcomes. Therefore, interventions resulting in overall Notch inhibition might be a promising

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Table 2. Cox multivariate and univariate analysis of overall survival in human lung adenocarcinoma based on Notch pathway category expression

Notch pathway category	Multivariate ^a P value	β	Univariate P value	β
Ligands	0.0423	0.15	0.1005	0.12
Downstream effectors	0.8329	0.02	0.8218	0.02
Receptors	0.1136	-0.12	0.2443	-0.08
Proteases	0.6712	-0.03	0.8505	0.01
Transcription factors	0.3104	-0.08	0.8117	-0.02
Modulators, positive	0.2559	-0.09	0.2503	-0.08
Modulators, unknown	0.3753	0.07	0.3798	0.07
Modulators, negative	0.0002	-0.30	0.0005	-0.26

^aAge, gender, stage, differentiation, and each Notch pathway category were included in each multivariate Cox model analysis.

therapeutic strategy to improve survival in patients with lung cancer.

Discussion

Serial analysis of gene expression (SAGE) profiling of NSCLC 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 single-nucleotide polymorphisms in the cell lines used in the article (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 CSCs, 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 *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). *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 CSC subpopulation, it has been challenging to reproduce these findings. CD133 was first reported by Eramo and colleagues 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 CSC features. This raises concerns regarding the validity of specific stem cell markers across various lung cancer model systems. The potential

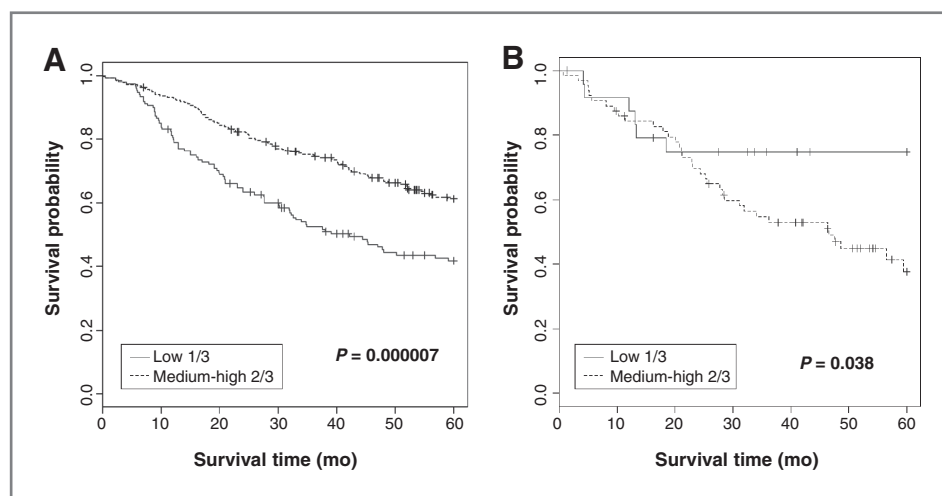


Figure 4. A, Kaplan-Meier curve of overall survival for patients with high and moderate expression of Notch-negative modulators compared with those with low expression. HR, 0.52; 95% confidence interval (CI), 0.39–0.70. B, Kaplan-Meier curve of overall survival for patients with high and moderate expression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

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 posttranslational 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 and colleagues indicating that Notch1 activity as determined by immunohistochemistry correlates with poor outcome in lung primary NSCLCs.

In the article, we delineated the role of Notch activity in lung CSCs 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.

Disclosure of Potential Conflicts of Interest

H. Korkaya has a commercial research grant from MedImmune. G.P. Kalemkerian has a commercial research grant from Lilly. M.S. Wicha has a

commercial research grant from MedImmune and Dompe, has ownership interest as cofounder of OncoMed Pharmaceuticals, and is a consultant for MedImmune, Verastem, and Paganini. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: K.A. Hassan, L. Wang

Development of methodology: K.A. Hassan, L. Wang, H. Korkaya, I. Maillard

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Hassan, L. Wang, D.G. Beer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.A. Hassan, L. Wang, G. Chen, I. Maillard, G.P. Kalemkerian, M.S. Wicha

Writing, review, and/or revision of the manuscript: K.A. Hassan, L. Wang, D.G. Beer, G.P. Kalemkerian, M.S. Wicha

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.A. Hassan, L. Wang, H. Korkaya
Study supervision: K.A. Hassan, G.P. Kalemkerian

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