Notch Reporter Activity in Breast Cancer Cell Lines Identifies a Subset of Cells with Stem Cell Activity

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Abstract

Developmental pathways such as Notch play a pivotal role in tissue-specific stem cell self-renewal as well as in tumor development. However, the role of Notch signaling in breast cancer stem cells (CSC) remains to be determined. We utilized a lentiviral Notch reporter system to identify a subset of cells with a higher Notch activity (Notch+) or reduced activity (Notch−) in multiple breast cancer cell lines. Using in vitro and mouse xenotransplantation assays, we investigated the role of the Notch pathway in breast CSC regulation. Breast cancer cells with increased Notch activity displayed increased sphere formation as well as expression of breast CSC markers. Interestingly Notch+ cells displayed higher Notch4 expression in both basal and luminal breast cancer cell lines. Moreover, Notch+ cells demonstrated tumor initiation capacity at serial dilutions in mouse xenografts, whereas Notch−cells failed to generate tumors. γ-Secretase inhibitor (GSI), a Notch blocker but not a chemotherapeutic agent, effectively targets these Notch+ cells in vitro and in mouse xenografts. Furthermore, elevated Notch4 and Hey1 expression in primary patient samples correlated with poor patient survival. Our study revealed a molecular mechanism for the role of Notch-mediated regulation of breast CSCs and provided a compelling rationale for CSC-targeted therapeutics. Mol Cancer Ther; 14(3); 779–87. ©2015 AACR.

Introduction

An increasing body of evidence suggests that a variety of cancers, including those of the breast, may be driven by a component of tumor-initiating cells that retain stem cell properties. Consistent with the cancer stem cell (CSC) model, the subset of tumor-initiating cells is able to generate tumors that recapitulate the phenotypic heterogeneity of the initial tumor. Recent studies, utilizing in situ “lineage tracing,” have demonstrated that the CSCs originate from tissue-specific stem cells in mouse models of skin, gut, and brain cancers (1–3). These studies provide further evidence that self-renewing tissue-specific stem cells may initiate and maintain tumors, mediate metastasis contributing to treatment resistance and relapse. Developmental pathways such as Notch are known to regulate self-renewal of embryonic and tissue-specific stem cells (4, 5). In addition, aberrant Notch signaling is associated with several human diseases, including malignant transformation of the mammary gland (6). In line with these findings, transactivation of Notch4 (formerly known as Int-3) as a consequence of insertional mutagenesis by the mouse mammary tumor virus results in malignant transformation of mouse mammary gland (6). These and in vitro studies lend strong support for a role of Notch in the regulation of CSCs.

Notch maintains pluripotent hematopoietic stem cells (HSC) by inhibiting differentiation (7). Consistent with these findings, the Notch pathway is linked to estrogen receptor (ER)-negative human breast tumors with a basal phenotype (8–11) suggesting a restriction in differentiation. Furthermore, we previously provided evidence that Notch induces self-renewal in mammary stem/progenitor cells (5). It has also been reported that Notch induces luminal differentiation of mouse mammary stem cells (12).

To investigate the role of Notch in breast CSCs, we utilized a Notch reporter system that allowed us to identify cells with Notch activity. Cells with Notch activity is isolated by flow cytometry and examined for self-renewal capacity utilizing in vitro CSC assays and stem cell markers such as aldehyde dehydrogenase (ALDH). In addition, we determined the tumor-initiating capacity of Notch-positive cells in mouse xenografts. Furthermore, we provide evidence that γ-secretase inhibitor (GSI) effectively targets Notch-positive cells and reduces tumor growth in mouse xenografts. Together these studies reveal a direct role for the Notch pathway in the regulation of breast CSCs and suggest that Notch-targeted therapeutics may be an attractive CSC-specific approach in treatment of breast cancer. In addition, our studies provide molecular mechanism for previous clinical findings that demonstrated the clinical efficacy of GSI in combination with docetaxel, a chemotherapeutic agent in patients with breast cancer.

References

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Materials and Methods

Cell lines and reagents

The breast cancer cell lines MDA-MB 231, MDA-MB 436, ZR-75-1, MCF7, ZR-75-30, and T47D were obtained from the ATCC more than 5 years ago and maintained in culture conditions as recommended by the ATCC. The Sum159 cell line was kindly provided by Dr. Stephen P. Ethier (Medical University of South Carolina, Charleston, SC). All cell lines were authenticated by the STR DNA profiling by the University of Michigan (Ann Arbor, MI) DNA sequencing core in 2011, before our studies presented in this article.

The GSI MRK-003 was kindly provided by Merck&Co., Inc., and stocks at 10 mmol/L in DMSO were used in in vitro studies. Docetaxel was purchased from Sanofi-Aventis.

Results of in vitro experiments are presented as mean ± standard deviation (SD) or mean ± standard error (SE) of three independent experiments. A Student t test was used to compare continuous variables. Median time to tumor formation was analyzed using the log rank test and the Kaplan–Meier method. SPSS version 13 was used for statistical analysis. P values of less than 0.05 were considered statistically significant.

Notch reporter lentiviral construct

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 were purchased from System Biosciences. Lentiviral production was performed by the University of Michigan Vector Core facility. Infection efficiency of cells with lentivirus was optimized using the positive Lentivirus expressing GFP under the control of CMV promoter and the multiplicity of infection has been determined to be 10. The Notch agonist Delta-Serrate-Lag (DSL) peptide was synthesized by the University of Michigan Peptide Core facility as previously described (5).

Mammosphere assay

Dissociated single MCF7-Notch and MCF7-mCMV cells were plated on 6-well ultra-low cluster plates (Corning Inc.) at a density of 1 × 105 cells/mL and grown for 7 to 10 days. Subsequent cultures after dissociation of primary spheres were plated on new plates at a density of 1 × 104 cells/mL. Mammosphere cultures were grown in a serum-free mammary epithelium basal medium as previously described (13).

In vivo tumorigenicity and MRK-003 treatments

Drug solutions were prepared fresh before each dose using a dounce homogenizer. MRK-003 (Merck Research Laboratories) was synthesized according to standard medicinal chemistry procedures. For the in vitro experiments, stocks were prepared at 10 mmol/L in DMSO and dilutions were made directly before use. For the in vivo experiments, MRK-003 was dosed as a suspension in 0.5% methylcellulose at a 10 mL/kg dosing volume, made fresh daily. Before administering each dose, the compound was thoroughly mixed to distribute suspension evenly. The dosing schedule for MRK-003 was 75 mg/kg by oral gavage, once daily using intermediate schedule (3 days on, 4 days off- four cycles), which was well tolerated.

Taxotere was dosed as a solution in 0.9% normal saline at a 10 mg/kg solution, intravenously, once per week.

All mice were housed in the AAALAC-accredited specific pathogen-free rodent facilities at the University of Michigan. Mice were housed in sterilized, ventilated racks, and supplied with commercial chow and sterile water both previously autoclaved. All experimentation involving live mice were conducted in accordance with standard operating procedures approved by the University Committee on the Use and Care of Animals at the University of Michigan. Athymic 4- to 6-week-old NOD/SCID mice were used. Single cells were flow cytometry sorted based on GFP expression and implanted into mammary fat pads. Mice were monitored via bioluminescence imaging over the course of 10 weeks utilizing the in vivo bioluminescence Caliper IVIS imaging systems. 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-nm filter. The single cells obtained were labeled with H-2Kd antibody and DAPI and then sorted with flow cytometry. Alive human cells were reincultured 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 × width²)/2.

RNA extraction and real-time RT-PCR

Total RNA was extracted using RNeasy Mini kit (QIAGEN) and 500 ng of RNA was used for making cDNA using Reverse Transcription System (Promega). Each cDNA was analyzed in triplicate using real-time qRT-PCR assays in an ABI PRISM 7900HT sequence detection system with 384-well block module and automation accessory (Applied Biosystems). The information of the PCR primers and fluorogenic probes used is available on the Applied Biosystems website (GAPDH: Hs00266705, Notch1: Hs01062014_m1, Notch2: Hs01050702_m1, Notch3: Hs01128541_m1, Notch4: Hs00965889_m1). The relative expression mRNA level was normalized against the internal control GAPDH gene [ΔCt = Ct (target gene) – Ct (GAPDH)]. The relative fold change was measured by 2-ΔΔCt formula compared with SUM159-Notch- control cells.

In vivo experiments

Mouse xenograft experiments are presented with mean and SE. Experiments with multiple tumors per mouse had standard errors of the mean calculated using clustering methods. Tumor growth experiments were analyzed using linear mixed models to account for the repeated measures. Correlation structures were dependent upon the experimental design. Luciferase experiments were assumed to have a first order autoregressive correlation structure. Pairwise comparisons were made within the models at cross-sections and the Bonferroni multiple comparisons adjustment was used for each experiment. Analyses were completed using SAS 9.3 (SAS Institute) with α of 0.05 determining statistical significance.

Immunofluorescence

Notch⁺ and Notch⁻ FACS-sorted cells were cytospun onto slides for immunofluorescence analysis. Slides were labeled with anti-human ALDH1A1 (ab51028, Abcam) or anti-human Hey1 (ab22614, Abcam) followed by species-specific alexafluor 555 nm-conjugated secondary antibody (Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) followed by species-specific alexafluor 488 nm-conjugated secondary antibody (Invitrogen).
labeled with DAPI (Sigma-Aldrich) and slides were mounted with mounting media for fluorescence (Vector Laboratories Inc.). Images were captured with a Zeiss LSM 510 laser scanning confocal microscope.

Flow cytometry
Cells were harvested in HBSS with 2% FBS, and GFP expression was measured on a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed using ModFit software (Verity Software House).

Results
Lentiviral Notch reporter system identifies a subset of breast cancer cells with increased Notch activity and CSC characteristics
Infection of multiple breast cancer cell lines with a lentiviral Notch reporter construct reveals a subset of cells with increased Notch activity (Notch+ cells) as determined by mCMV-driven GFP expression under the control of a Notch-specific transcriptional response element (Fig. 1A and B). Breast cancer cell lines, MDA-MB-231, SUM159, and MDA-MB-436, representing the basal-like or claudin-low subtype contained a higher Notch activity as compared with cell lines, ZR-75-1, MCF7, ZR-75-30, and T47D, which represent the luminal subtype (Fig. 1B and C).

To determine whether the CSC marker, ALDH1 expression overlaps with the Notch activity (14), Notch-reporter expressing cells were cytospun and stained with ALDH1 antibody. ALDH1 expression was primarily found in GFP-expressing Notch+ cells (Fig. 2A), suggesting a higher Notch activity in breast CSCs. There was also a statistically significant overlap between Notch activity and CD44+/CD24− phenotype (Fig. 1B). In agreement with CSC properties, we demonstrated that the Notch+ cells possessed a higher sphere forming capacity as compared with Notch− cells (Fig. 2C). Further analyses of these cells revealed that Notch+ cells had reduced levels of ER expression compared with the Notch− cells (Fig. 2D). To determine whether these Notch+ cells are responsive to Notch stimulation, the Notch agonist peptide, DSL was used to activate the Notch pathway leading to increased GFP expression and expansion of Notch+ cell population (Fig. 2E). This was further confirmed with dose-dependent activation of Notch reporter upon stimulation of cells with recombinant DLL4 (Fig. 2F). Endothelial cells were reported to have higher Notch ligands particularly DLL4 expression (15). Consistent with these reports, there was a time-dependent increase in the Notch activity when Notch reporter-expressing MCF7 cells were cocultured with endothelial HUVEC cells (Fig. 2G).

Notch activity correlates with higher Notch4 expression and downstream targets
To determine which Notch receptor may be responsible for Notch activity, we quantified the expression of Notch1, Notch2, Notch3, Notch4, and NICD in Notch reporter-expressing and control breast cancer cell lines. The expression levels of Notch receptors were measured using qPCR and were found to be significantly higher in Notch reporter-expressing cells as compared with control cells (Fig. 2H). Consistent with these results, we observed an increase in the expression of the downstream target gene, Hes1, which encodes a transcriptional repressor of the Hes family (16). These findings suggest that Notch signaling is an important mechanism for the maintenance of breast CSCs.
Notch2, Notch3, and Notch4 in Notch+ and Notch− cells. qPCR experiments demonstrated that Notch4 receptor was expressed at 3- to 5-fold higher levels in the Notch+ cells than the Notch− cells (Fig. 3A). We next examined the expression of Notch-specific ligands and downstream targets (16) by qPCR and immunofluorescent experiments. Downstream effectors Hey1 but not Hes1 are significantly higher in Notch+ cells compared with the Notch− cells (Fig. 3B and C). Expressions of the Notch ligands DLL1 and Jagged1 were significantly higher in Notch+ cells as compared with the Notch− cells (Fig. 2B). We reasoned whether higher Notch4 expression was correlated with these ligands and downstream targets as well as with patient survival. We utilized The cancer genome atlas (TCGA) dataset (1,061 patient samples) submitted to free cBioPortal website (17) and demonstrated that there is indeed a positive correlation between the Notch4 mRNA expression and DLL1, DLL4, Jagged1, and Hey1 mRNA expressions (Supplementary Fig. S1A and S1B). It has been reported that Notch is required for Wnt-mediated malignant transformation of mammary epithelial cells (18). Moreover our previous studies demonstrated that the Wnt/β-catenin pathway is selectively activated in breast CSCs (19). To demonstrate a possible link between the Notch and Wnt/β-catenin pathways, FACS-sorted Notch+ and Notch− cells were cytopun on slides and stained for β-catenin. Notch+ cells displayed a higher β-catenin nuclear localization compared with the Notch− cells (Fig. 3D), suggesting a link between these two pathways. Furthermore, elevated Notch4-Hey1 mRNA expressions but not Notch3-Hey1 correlated with poor patient survival as demonstrated by using the cBioPortal (Fig. 3E and F). These results strongly suggest that Notch4 receptor plays a major role in Notch activity and correlates with expression of ligands and downstream effectors and appears to have a clinical relevance in patient samples.

Single Notch+ cells, but not Notch− cells, have the capacity to repopulate initial heterogeneity

Like their normal counterparts, the hallmark of CSCs has been defined as self-renewal and lineage differentiation (20). To provide further evidence that Notch+ cells possess self-renewing and lineage differentiation capacity, we established multiple independent clones from Notch+ and Notch− cells. We first ensured that all the cells had the Notch reporter construct by presorting Notch+ cells in bulk. These Notch+ cells generated both Notch+ and Notch− cell populations in 2 weeks (Fig. 4A). However, FACS sorting in bulk is associated with 5% to 10% impurity (21) and does not eliminate the possibility of contamination of Notch−...
the Notch pathway is involved in breast CSC self-renewal and lineage specification. Although the Notch pathway has been shown to play a crucial role in breast CSC self-renewing capacity and generate tumors in mouse xenografts, the Notch activity is involved in breast CSC self-renewal remains unknown. To determine whether the Notch pathway drives the expression of GFP and luciferase genes under the Notch-responsive element, and thus the luciferase expression is utilized to monitor Notch activity and tumor growth in live animals. As outlined in Supplementary Fig. S2, primary Sum159 and MCF7 tumors that express Notch reporter were dissociated, Notch+ or Notch- cells were FACS sorted and implanted into secondary mice to evaluate the ability of Notch+ or Notch- cells to form tumors. Notch+ Sum159 (50K) and MCF7 (100K) cells formed significantly larger tumors with shorter latency periods compared with Notch- cells (Fig. 5A and C and Supplementary Fig. S2). Although Notch+ tumors were small (and precluded the FACS analyses), both Notch+ Sum159 and MCF7 tumors displayed initial phenotypic heterogeneity by generating both Notch+ cells as well as Notch- cells (Fig. 5B and D). We next assessed the CSC frequency of Notch+ and Notch- cells in limiting-dilution transplantation assays in tertiary mouse xenografts. Limiting dilutions of Notch+ or Notch- negative cells (from Sum159 or MCF7 cell lines) were implanted and the tumor growths were determined in each group at 10 weeks. These tertiary limiting-dilution xenotransplantation assays demonstrated that the CSC frequency was significantly higher in Notch+ cells compared with Notch- cells from both Sum159 or MCF7 cell lines (Tables 1 and 2), suggesting a higher Notch activity in the CSC population. Furthermore, Notch+ cells meet the criteria of CSC features by generating tumors in a serial transplantation assays and differentiating into Notch- cell population.

**Figure 3.**

Notch reporter-positive cells express higher Notch1, Notch2, and Notch4 receptors and downstream effectors compared with the negative cells. A and B, qPCR analyses show increased expressions of Notch1, 2, and 4, Notch ligands Jagged 1 and DLL1, and downstream effector Hey1 in Notch+ cells compared with Notch- cell populations. Notch+ cells compared with the Notch- cells display higher Hey1 expression (C) and increased nuclear β-catenin localization (D) as assessed by IF. E and F, analyses of TCGA breast cancer dataset (CBioPortal) revealed that elevated Notch4/Hey1 expressions, but not Notch2/Hey1 expressions, correlated with poor patient survival. Mean ± SD (n = 3), *, P ≤ 0.05; **, P ≤ 0.005.
As demonstrated in Table 1, Sum159 Notch+ cells display a higher CSC frequency (1 in 67) compared with the Notch− cell population. Thus, we investigated the ability of single Notch+ cells to generate tumors by implanting single Notch+ or single Notch− cells into the fat pads of NOD-SCID mice. As expected from limiting-dilution transplantation experiments, single Notch− cell implantations failed to generate tumors in mice (Supplementary Fig. S3A, left fat pads). In contrast, one out of ten single Notch+ cell implantations generated tumors as demonstrated by bioluminescence imaging starting 4 weeks postinjection (Supplementary Fig. S3A, right fat pads). We evaluated this single Notch+ cell-generated tumor to determine the ability of a single cell reconstituting the initial phenotypic heterogeneity. FACS analyses revealed that a single Notch+ cell was able to generate both Notch+ and Notch− cell populations as demonstrated by GFP expression (Supplementary Fig. S3B). Targeting Notch with GSI inhibits Notch activity and reduces the tumor-initiating capacity of breast cancer cells

Recent preclinical and clinical studies suggest that the efficacy of GSI in patients with breast cancer may be due to its effect on the breast CSCs (22–24). Our studies provide evidence that the Notch-reporter system with luciferase expression may be an attractive tool to monitor Notch activity in growing tumors in live animals. Therefore, we utilized GSI to determine whether the inhibition of Notch would efficiently target breast CSCs and inhibit tumor growth. We implanted the Notch-reporter expressing Sum159 cells into the fat pads of NOD-SCID mice and treated them with two cycles of GSI alone or in combination with a chemotherapeutic agent (docetaxel) at 3 weeks postimplantation (Supplementary Fig. S4). We measured the tumor size over the course of the study and determined not surprisingly that GSI treatment did not have much effect on primary tumor size. However, Notch activity was significantly diminished after the second cycle of GSI alone or GSI plus docetaxel treatment as measured by luciferase imaging in live animals (Fig. 6A). Furthermore, analyses of these tumors by flow cytometry also confirmed that Notch-driven GFP expression was significantly reduced in GSI-treated tumors (Fig. 6B). Therefore, we reasoned that inhibition of Notch activity by GSI in primary tumors may target CSCs and if so these tumors should have reduced tumor-initiating capacity in secondary reimplantations at limiting dilutions. Thus, the primary tumors from mice that were either untreated or treated with docetaxel or GSI alone or in combination were reimplanted at indicated dilutions into the fat pads of secondary mice. Following 10 weeks of monitoring, we determined that the GSI alone or GSI in combination with docetaxel-treated tumors not only displayed significantly lower tumor-initiating capacity, but also had reduced CSC frequency (1 in 504 in GSI alone, 1 in 1,602 in GSI+docetaxel compared with 1 in 23 in control and 1 in 88 in docetaxel-treated tumors; Fig. 6C and Table 3).
Subject: Notch Activity Identifies Breast Cancer Stem Cells

Discussion

Despite recent advances in biology and improved diagnostics, intrinsic and acquired resistance to endocrine, trastuzumab, or other chemotherapeutic agents, remains one of the most significant challenges for treatment of metastatic breast cancer (25, 26). Clinical implications of the CSC concept and the role of these cells in mediating the tumorigenesis suggest that these cells might provide a promising therapeutic target (27, 28). Furthermore, accumulating evidence now from a number of solid tumors suggests that CSCs are more resistant to chemotherapy and radiation therapy as compared with bulk tumor cells (28–32). The Notch pathway has been implicated in mediating resistance to chemotherapeutic agents in many human malignancies, including breast (22, 33, 34). Utilizing a novel Notch reporter system, we investigated its direct role in tumor initiation and therapeutic resistance in in vitro assays and in mouse xenografts models. In agreement with previous loss of function studies (35), we demonstrated that breast cancer cells with higher Notch activity indeed display stem cell features and expression of CSC markers (14). As previously reported (36), we found higher Notch activity in basal-like breast cancer cell lines compared with luminal breast cancer cell lines. Interestingly Notch4 receptor expression 3- to 5-fold higher in Notch $^+$ cells compared with Notch $^-$ cells, suggesting a major role on the Notch pathway.

The Notch reporter system has been shown to be a useful tool to monitor Notch activity in developing organs in live animals as well as in lung tumor xenografts (37, 38). Thus using this reporter system, we determined that Notch $^+$ cells had significantly higher tumor-initiating capacity compared with Notch $^-$ cells in serial transplantation assays. Primary and secondary tumors generated by Notch $^+$ cells had the in vivo lineage differentiation capacity since they generated both Notch $^+$ and Notch $^-$ cells. Limiting-dilution transplantation assays demonstrated a significantly higher CSC frequency within the Notch $^+$ cell population (1 in 67) compared with the Notch $^-$ cell population (1 in 9,848). Together these findings provide strong evidence that Notch activity substantially enriches breast CSCs in vivo and thus may have

Table 1. Tertiary tumor growth and the CSC frequency of Notch $^+$ and Notch $^-$ Sum159 breast cancer cell subpopulations in NOD-SCID mice using the serial transplantation assay

<table>
<thead>
<tr>
<th>Cell populations</th>
<th>Cell number</th>
<th>Tumors/number of implantations</th>
<th>CSC frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum159-Notch $^+$</td>
<td>15,000</td>
<td>6/6</td>
<td>Notch $^+$ 67</td>
</tr>
<tr>
<td>Sum159-Notch $^-$</td>
<td>1,000</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Sum159-Notch $^+$</td>
<td>500</td>
<td>2/3</td>
<td></td>
</tr>
<tr>
<td>Sum159-Notch $^-$</td>
<td>100</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>Sum159-Notch $^+$</td>
<td>15,000</td>
<td>2/6</td>
<td>Notch $^+$ 9848</td>
</tr>
<tr>
<td>Sum159-Notch $^-$</td>
<td>1,000</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Sum159-Notch $^+$</td>
<td>500</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Sum159-Notch $^-$</td>
<td>100</td>
<td>0/3</td>
<td></td>
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</tbody>
</table>

Note: $P = 0.000000199$.
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therapeutic utility. Elevated expressions of Notch4 and Hey1 significantly correlated with poor patient survival among patients with breast cancer, further supporting the clinical relevance of the Notch pathway.

In early-phase clinical trials, targeting Notch in patients with breast cancer with GSI provided a clinical benefit which may be explained by its CSC-specific activity (22). To provide a more direct molecular role for Notch in mouse xenografts, we treated mice bearing the Notch reporter expressing Sum159 cells and monitored Notch activity in growing tumors in live animals. Two cycles of GSI treatment reduced the Notch activity and reduced the CSC population. This activity was further enhanced when combined with docetaxel because there was no tumor growth in secondary tumor implantations.

In summary, our studies provide direct evidence for a role of the Notch pathway in CSC self-renewal and tumor initiation in in vitro assays and in live animals by utilizing a novel Notch reporter system. Together with previous reports, it further emphasizes the importance of CSC-targeted therapeutics in cancer treatments.

Disclosure of Potential Conflicts of Interest

M.S. Wicha reports receiving commercial research grants from MedImmune and Dompe Pharma, has ownership interest (including patents) in OncoMed, and is a consultant/advisory board member for MedImmune, Verastem, Paganini, and Cerulean. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: H. Korkaya, R.C. D’Angelo, M.S. Wicha
Development of methodology: H. Korkaya, R.C. D’Angelo, T. Luther, M.S. Wicha
Acquisition of data (providing materials, acquiring and managed patients, provided facilities, etc.): H. Korkaya, R.C. D’Angelo, M. Ouzounova, A. Davis, D. Choi, G. Kim, T. Luther, A.A. Quraishi, S.J. Conley, K.A. Hassan, S.G. Clouthier, S.M. Tchuenkam
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Korkaya, R.C. D’Angelo, M. Ouzounova, D. Choi, Y. Senbahaorglu, S.J. Conley, M.S. Wicha
Writing, review, and/or revision of the manuscript: H. Korkaya, R.C. D’Angelo, M. Ouzounova, M.S. Wicha, S.G. Clouthier

Table 3. Residual tumor growth following indicated treatments and the CSC frequency estimated in reimplantation assays in NOD-SCID mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of cells</th>
<th>Tumors/implantations</th>
<th>CSC frequency</th>
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<td>23</td>
</tr>
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<td>Control</td>
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<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>GSI</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Docetaxel</td>
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<td>Docetaxel</td>
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</tr>
<tr>
<td>Docetaxel</td>
<td>100</td>
<td>2/3</td>
<td></td>
</tr>
<tr>
<td>GSI + docetaxel</td>
<td>1,000</td>
<td>0/3</td>
<td>1,602</td>
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<tr>
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<td>500</td>
<td>0/3</td>
<td></td>
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<tr>
<td>GSI + docetaxel</td>
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<tr>
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<td>Control</td>
<td>GSI</td>
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<tr>
<td>Control</td>
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<td>GSI</td>
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<tr>
<td>GSI</td>
<td>GSI + docetaxel</td>
<td>0.0813</td>
</tr>
</tbody>
</table>

In summary, our studies provide direct evidence for a role of the Notch pathway in CSC self-renewal and tumor initiation in in vitro assays and in live animals by utilizing a novel Notch reporter system. Together with previous reports, it further emphasizes the importance of CSC-targeted therapeutics in cancer treatments.
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Study supervision: H. Korkaya

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