Companion Diagnostics and Cancer Biomarkers

Molecular Cancer Therapeutics

Coordinate Hyperactivation of Notch1 and Ras/MAPK Pathways Correlates with Poor Patient Survival: Novel Therapeutic Strategy for Aggressive Breast Cancers

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Abstract

Aberrant activation of Notch and Ras pathways has been detected in breast cancers. A synergy between these two pathways has also been shown in breast cell transformation in culture. Yet, the clinical relevance of Notch–Ras cooperation in breast cancer progression remains unexplored. In this study, we show that coordinate hyperactivation of Notch1 and Ras/MAPK pathways in breast cancer patient specimens, as assessed by IHC for cleaved Notch1 and pErk1/2, respectively, correlated with early relapse to vital organs and poor overall survival. Interestingly, majority of such Notch1^{high}Erk^{high} cases encompassed the highly aggressive triple-negative breast cancers (TNBC), and were enriched in stem cell markers. We further show that combinatorial inhibition of Notch1 and Ras/MAPK pathways, using a novel mAb against Notch1 and a MEK inhibitor, respectively, led to a significant reduction in proliferation and survival of breast cancer cells compared with individual inhibition. Combined inhibition also abrogated sphere-forming potential, and depleted the putative cancer stem-like cell subpopulation. Most importantly, combinatorial inhibition of Notch1 and Ras/MAPK pathways completely blocked tumor growth in a panel of breast cancer xenografts, including the TNBCs. Thus, our study identifies coordinate hyperactivation of Notch1 and Ras/MAPK pathways as novel biomarkers for poor breast cancer outcome. Furthermore, based on our preclinical data, we propose combinatorial targeting of these two pathways as a treatment strategy for highly aggressive breast cancers, particularly the TNBCs that currently lack any targeted therapeutic module. *Mol Cancer Ther; 13(12); 3198–209. ©2014 AACR.*

Introduction

Breast cancer remains a major health burden affecting the lives of millions of women the world over. International Agency for Research on Cancer data reported that 1.7 million women were diagnosed with breast cancer worldwide in 2012. In India, the incidence of breast cancer is on the rise (1), and has surpassed cervical cancer in the metropolitan cities. The chances of disease-free survival of patients with breast cancer have increased over the last few decades; however, this is applicable only if the disease is diagnosed at an early stage and is limited to the primary organ site (2). Once breast cancer metastasizes to other organs, the therapeutic options are very limited and the success rate of managing such patients in the clinics is poor. Therefore, there is an urgent need for the development of mechanism-based, targeted therapeutic strategies with improved outcomes for the treatment of aggressive cancers.

Breast cancer is a heterogeneous disease that can be classified using a variety of clinical and pathologic features. The status of three hormone receptors—estrogen receptor (ER), progesterone receptor (PR), and HER2—is routinely used to categorize breast cancers and they also serve as predictive biomarkers to select specific adjuvant therapies (3). ER$^+$ and/or PR$^+$ tumors are administered with hormone therapy and in general show good survival rates. Similarly, HER2 positivity is useful for selecting targeted therapy with mAb (trastuzumab) against HER2 (4). In contrast, the triple-negative breast cancer (TNBC) subtype, which accounts for approximately 15% of breast cancer cases, is characterized by the absence of ER, PR, and HER2. As a group, these tumors exhibit an aggressive clinical phenotype with early development of visceral metastases and a poor long-term prognosis (5). Thus, the TNBCs constitute an imperative clinical challenge, as they do not

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respond to endocrine treatment and currently lack any targeted therapies.

Notch signaling is an evolutionary conserved pathway and plays a key role in various cell-fate decisions throughout embryonic development and later in adult homeostasis (6). There are four Notch receptors and five ligands; interactions between Notch receptor and ligand result in proteolytic cleavages finally leading to the release of the Notch intracellular domain (NICD) that translocates to the nucleus and functions as a transcription factor (7). Aberrant Notch signaling has been associated with various cancers, including breast cancers (8, 9). High-level expression of Notch1 is an early event during breast carcinogenesis, and together with a ligand Jagged1, is predictive of poor overall survival (OS; ref. 10). We have previously demonstrated overexpression of several Notch receptors and ligands in breast cancers, as well as Notch activation in early precursors and invasive ductal carcinoma (11). Recent reports have associated Notch signaling with TNBCs (12) and aggressive phenotypes (13). Furthermore, recent studies have revealed the requirement of Notch signaling for the maintenance of breast cancer stem-like cells (14, 15). Thus, Notch signaling has been associated with both the development and progression of breast cancers, suggesting that inhibition of Notch signaling may be beneficial for breast cancer treatment (16).

The oncogenic functions of Notch signaling in breast cancers may be mediated through its cross-talks with other signaling pathways. For example, cross-talk between Notch and ER signaling has been documented in breast cancers (17). Furthermore, cross-talk between ErbB1/2 and Notch signaling has been shown in ductal carcinoma *in situ*, and combined inhibition of these two pathways was found to be more effective in targeting breast cancer cells (18). Even though Ras mutations are not that common in breast cancers, multiple receptor tyrosine kinases associated with breast cancers activate Ras, which results in the activation of the MAPK cascade involving Raf, MEK, and MAPK (Erk1/2; ref. 19). Ras overexpression/activation led to upregulation of Notch1 (20), and Notch-mediated oncogenesis requires Ras pathway signals (21), suggesting an association between these two pathways in breast cancer pathogenesis. Consistent with this, we demonstrated a cooperation between active Notch1 and Ras/MAPK pathways in mediating cellular transformation of immortalized breast cells (11). Interestingly, cleaved (active) Notch1–positive tumors with increased expression of phosphoErk1/2 (active MAPK) showed high node positivity (11), suggesting that Notch–Ras/MAPK cooperation may lead to more aggressive disease. However, the clinical relevance of Notch–Ras coactivation has not been explored thus far.

In this study, we sought to comprehend the consequences of coordinate activation of Notch1 and Ras/MAPK pathways on the survival of breast cancer patients, and the efficacy of their combinatorial inhibition on breast cancer cell lines. We report, here, that coordinate hyperactivation of Notch1 and Ras/MAPK pathways correlates with poor OS in patients with breast cancer. Majority of such cases encompassed the TNBCs and were enriched in stem cell markers like Oct4, Nanog, and CD44. Simultaneous inhibition of Notch1 and Ras/MAPK with MAb602.101 and MEK inhibitor PD98059, respectively, resulted in a significant reduction in proliferation and survival, and abrogation of mammosphere formation. Importantly, combined inhibition led to a depletion of the stem-cell like population of breast cancer cells *in vitro*, and blocked tumor growth *in vivo*. Taken together, our study demonstrates a nexus between Notch1 and Ras/MAPK signaling in aggressive breast cancers, including TNBCs, and provides promising preclinical data to target these cancers by combinatorial inhibition of these two pathways.

Materials and Methods

Cell lines

Breast cancer cell lines BT-474, MCF-7, MDA-MB-231, and HCC-1806 (obtained from the ATCC and no further authentication was performed in the past 6 months) were cultured in DMEM (Sigma) supplemented with 10% FBS (Invitrogen), NBLE cells (described in ref. 22) and primary cancer tissue–derived cells were cultured in serum-free DMEM-F12 media supplemented with growth factors (22) and maintained under standard tissue culture conditions of 37°C in a humidified incubator.

IHC and tissue samples

Breast cancer tissue sections were obtained from tumor blocks archived in the Department of Pathology at the Kidwai Memorial Institute of Oncology (KMIO; Bangalore, KA, India). IHC was performed as described previously (11) using cleaved Notch1–specific antibody (2421 V1744 and CST) that detects active Notch1 generated following γ-secretase cleavage, pErk1/2 (C33E10; CST), Oct4 (ab19857; Abcam), CD44 (3570S; CST), Nanog (SC-33759; Santa Cruz Biotechnology). Immunohistochemical intensity and distribution were semiquantitatively scored by an experienced pathologist (R.V. Kumar) using the Allred score method for the nuclear staining, and the membrane and cytoplasmic staining was scored on a relative scale as described previously (11). For mammosphere formation assays, primary breast tumor tissue was obtained with patient consent from KMIO, as approved by the Medical Ethics Committee (Institutional Review Board of KMIO) and in compliance with the ethical guidelines of Indian Institute of Science (IISc; Bangalore, KA, India). The primary tissues were processed as described previously (22).

Patient follow-up data and analysis

Clinical and follow-up data (from 3 to 9 years) of 115 patients with breast cancer with invasive ductal carcinoma grade 3 were collected from the medical records of KMIO, with informed patient consent. This study was

approved by the Medical Ethical Committee, KMIO, and Ethics committee of the IISc. The OS was measured from diagnosis to death or last date of follow-up. Kaplan–Meier curves were calculated for the high and low expression of cleaved Notch1 and pErk1/2 groups.

Cell proliferation, viability, and apoptosis assays

To investigate the effects of Notch1 inhibition, we used anti-Notch1 MAb602.101 described previously (15), and for Ras/MAPK inhibition, we used MEK inhibitor PD98059 (CST). For proliferation assays a panel of breast cancer cell lines MDA-MB-231, HCC-1806, BT-474, and MCF-7 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated with MAb602.102 and PD98059, alone or combination, in a dose-dependent manner for 72 hours. Cells were subsequently treated with bromodeoxyuridine (BrdUrd; Calbiochem) for 12 hours and its incorporation determined as per the protocol recommended by the manufacturer. Cell viability was evaluated by using MTT (Sigma) after 48 hours of treatment with PD98059 and MAb602.101, alone or in combination, in a dose-dependent manner, followed by analysis of formazan formation at absorbance of 550 nm using ELISA plate reader. Apoptotic cell death was assessed by incubating cells with PD98059 (10 μmol/L) and MAb602.101 (10 μg/mL) followed by staining with Annexin V–PE-Cy5 and analyzed by flow cytometry.

Analysis of putative breast cancer stem cells ($CD44^{high}/CD24^{low}$) subpopulation

Breast cancer cells (1×10^5) treated for 48 hours with PD98059 (10 μmol/L) and MAb602.101(10 μg/mL), individually or in combination, were harvested using trypsin-EDTA, resuspended in Dulbecco's Phosphate-Buffered Saline (DPBS) containing 2% FBS (FBS/PBS), and incubated with anti–CD44-FITC–(BD-555478) and anti–CD24-PE (BD-555428)–conjugated primary antibodies for 45 minutes at 4°C on ice with intermittent mixing, followed by washing, resuspension in DPBS, and analysis using Becton Dickinson FACS canto. The percentage of cells in each quadrant was calculated using the Stat program of Cell Quest by Becton Dickinson.

Sphere formation assay

The breast cancer cell lines MDA-MB-231, HCC-1806, MCF-7, and BT-474 (1×10^5 cells/well of a 6-well plate) were subjected to sphere formation by seeding in a semi-solid medium containing 1.5% methylcellulose. The NBLE cells and enzymatically dissociated single-cell suspensions of the primary breast cancer tissues (2×10^4 cells/well of a 6-well plate) were seeded in ultra-low attachment plates in serum-free DMEM-F12 supplemented with growth factors. Mammospheres were formed between 7 and 10 days and were counted under the microscope as described previously (22, 23). Effect of PD98059 and MAb602.101 on sphere-forming efficiency of these cells was assessed by incubating the cells with PD98059 (10 μmol/L) and MAb602.101 (10 μg/mL), alone or in combination.

Tumor xenograft assays

Mice experiments were undertaken with prior approval from the Animal Ethics Committee (IISc). HCC-1806, MDA-MB-231, and BT-474 (1×10^6) cells were injected s.c. into each flank of 5-week-old female nude mice. When tumors reached 100 mm^3 in volume, the mice were randomized into five groups and were treated with vehicle (DMSO), control IgG [15 mg/kg body weight (b.w.)], MAb602.101 (15 mg/kg b.w.) , PD98059 (50 μmol/L; ref. 24), and combination of MAb602.101 and PD98059. The MAb602.101 and control-IgG were administered i.p. whereas DMSO and PD98059 were administered intratumorally every 3 days. The treatment was given for a period of 2 weeks and tumor measurements were taken every 2 days for 2 weeks.

Statistical analyses

Statistical analyses were performed with the Fisher exact test, the Student *t* test, one-way ANOVA and survival analysis using graph-pad prism-5 software. Cox regression analysis was performed with SPSS-16 software. A P value of <0.05 was considered statistically significant.

Results

Coordinate hyperactivation of Notch1 and Ras/MAPK pathways is associated with increased risk of lymph node metastasis, early relapse, and poor OS

Our earlier study had revealed hyperactivation of Notch1 and Ras/MAPK in high node–positive grade 3 invasive ductal carcinoma (11), suggesting a possible association of Notch–Ras activation with increased breast tumor aggressiveness. To further explore this, we extended our study to a larger number (115) of patients with grade 3 ductal carcinoma breast cancer, and evaluated their status of Notch1–Ras/MAPK pathways, and additionally, the patient outcome (Table 1A). To do so, we undertook IHC analyses for Notch1 activation using cleaved Notch1 antibody that specifically detects the active form of Notch1 (NICD) generated by γ-secretase cleavage. To detect Ras/MAPK activation, we evaluated the phosphorylation status of Erk (MAPK) using pErk1/2-specific antibodies. Stainings were given relative grading based on the expression status of cleaved Notch1 and phosphoErk1/2, and categorized as $clNotch^{high}pErk^{high}$ (those expressing high levels of both cleaved Notch1 and pErk1/2) or the "rest" (including $clNotch^{low}pErk^{low}$, $clNotch^{high}pErk^{-/low}$, and $clNotch^{-/low}pErk^{high}$; Supplementary Fig. S1A).

Our IHC-based investigations revealed that 61.7% of these cases (71/115) showed a $clNotch^{high}pErk^{high}$ phenotype (Table 1A). Furthermore, we observed high expression of clNotch1 in 71 of 80 samples with high pErk expression, indicating a significant positive correlation between high pErk and clNotch1 expression ($P < 0.0001$, Table 1B). Of these 71 cases that showed $clNotch^{high}pErk^{high}$ phenotype,

Table 1.

A, Immunohistochemical and clinicopathologic parameters of breast cancer patient samples analyzed

Category based on cleaved Notch1 and pErk1/2 status	Number category /total number of patients	Number of LN^+/total category	Number of LN^- /total category	Number of patients with metastasis to vital organs/total category	Number of patients with metastasis to bone/total category	Number of patients free of metastasis /total category
clNotch1^{high} pErk^{high}	71/115 (61.7%) TNBC 45/71 (63.3%) Her2 17/71 (23.9%) ER/PR 9/71 (12.6%)	61/71 (84. 91%) TNBC 45/45 (100%) Her2 12/17 (70.58%) ER/PR 4/9 (44.4%)	10/71 (14.08%) TNBC 0 Her2 5 ER/PR 5	41/71 (57.74%) TNBC 34 Her2 6 ER/PR 1	19/71 (26.76%) TNBC 11 Her 2 6 ER/PR 2	11/71 (15.49%) TNBC 0 Her2 5 ER/PR 6
Rest clNotch1^{low} pErk^{low} clNotch1^{high} pErk^{low} clNotch1^{low} pErk^{high}	44/115 (38.26%) TNBC 10 Her2 12 ER/PR 22	15/44 (34.09%) TNBC 0 Her2 9 ER/PR 6	29/44 (65.9%) TNBC 10 Her2 3 ER/PR 16	8/44 (18.18%) TNBC 5 Her2 3 ER/PR 0	12/44 (27.27%) TNBC 4 Her2 6 ER/PR 2	24/44 (54.54%) TNBC 1 Her2 3 ER/PR 20

NOTE: A total of 115 patient samples of grade 3 invasive ductal carcinoma were analyzed for the expression of cleaved Notch1 and pErk1/2 and further subdivided into clNotch1high pErkhigh and "rest" categories and correlated with their ER/PR/Her2 status, node status, metastasis to vital organs and bone, and free of metastasis.

B, Correlation between clNotch1^{high} and pErk^{high}

Breast cancer patient samples		clNotch1 High	clNotch1 low	Total
pErk1/2^{high}	High	71	9	80
	Low	13	22	35
Total		84	31	115
$P < 0.0001$; OR, 13.35; 95% CI, 5.0 33–35.41				

C, Correlation between clNotch1^{high}/pErk^{high} and LN status

Breast cancer patient samples	LN status Positive	LN status Negative	Total
clNotch1^{high}pErk^{high}	61	10	71
Rest	15	29	44
Total	76	39	115
$P < 0.0001$; OR, 11.89; 95% CI, 4.726–29.43			

D, Correlation between clNotch1^{high}/pErk^{high} and metastasis

Breast cancer patient samples	Metastasis status Positive	Metastasis status Negative	Total
clNotch1^{high}pErk^{high}	60	11	71
Rest	20	24	44
Total	80	35	115
$P < 0.0001$; OR, 6.545; 95% CI, 2.728–15.70			

E, Cox regression analyses of clNotch1 and pErk expression, LN status, and metastasis in relation to the OS of patients with breast cancer

Variable	HR (95% CI)	*P*
Univariate analysis		
clNotch1^{high}pErk^{high}	2.148 (1.658–2.781)	<0.0001
clNotch1^{low}pErk^{low}	0.715 (0.438–1.660)	0.179 (NS)
clNotch1^{low}pErk^{high}	1.732 (1.299–2.309)	<0.0001
clNotch1^{high}pErk^{low}	1.952 (1.441–2.644)	<0.0001
LN status	6.374 (3.530–11.50)	<0.0001
Metastasis	4.426 (2.610–7.480)	<0.0001
Multivariate analysis		
clNotch1^{high}pErk^{high}	2.724 (1.478–5.019)	0.001
clNotch1^{high}pErk^{low}	0.651 (0.373–1.136)	0.131 (NS)
clNotch1^{low}pErk^{high}	0.842 (0.456–1.557)	0.583 (NS)
LN status	2.478 (1.425–4.308)	0.001
Metastasis	3.943 (2.137–7.275)	<0.0001

63.3% (45/71) were TNBCs, 23.9% (17/71) were Her2$^+$, and 12.6% (9/71) were ER/PR$^+$. Interestingly, a vast majority of the TNBCs, 83.8% (45/55), fell in the clNotch^{high}pErk^{high} category.

Because axillary lymph node (LN) status is one of the most important prognostic variables in the management of patients with primary breast cancer (25), we checked the node status of these cases. A vast majority of cases within the clNotch^{high}pErk^{high} category, 84.91% (61/71), exhibited LN metastasis (Table 1A and C), further supporting our previous observations (11), and suggesting that the status of Notch1 and Ras/MAPK pathway activation can serve as a prognostic marker. Interestingly, all the TNBCs within the clNotch^{high}pErk^{high} category (45/45) showed high node positivity (Table 1A), and additionally showed elevated expression of stemness markers like Oct4, nanog, and CD44 (Supplementary Fig. S1B), further suggesting a possible correlation between Notch1–Ras/MAPK activation, stemness, and tumor aggressiveness.

Analogous to several other cancers, the majority of deaths associated with breast cancers are due to metastatic growth and relapse that impairs the functions of vital organs like brain, liver, and lungs, thereby presenting a major clinical challenge for achieving disease-free survival (25). Our findings revealing the association of Notch1 and Ras/MAPK activation with aggressive breast cancers (Table 1A–C) was also suggestive of its plausible association with relapse. We therefore carried out an extensive and detailed follow-up of patients to investigate whether the expression status of clNotch1 and pErk correlated with overall patient survival. Our study revealed that a large number of patients within the clNotch^{high}pErk^{high} category had early relapse to vital organs, including the brain, lungs, and liver (41/71) and/or bone metastasis (19/71; Table 1A and D). A multivariate analysis undertaken by entering the significant variables from the univariate analyses revealed that clNotch^{high}pErk^{high} expression, LN status, and metastasis were associated with OS ($P < 0.001$; Table 1E). Furthermore, the Kaplan–Meier survival analyses revealed poor OS rates in patients with clNotch^{high} pErk^{high} phenotype compared with the "rest" category (Fig. 1A). Interestingly, this trend held true across various breast cancer subtypes, including ER/PR$^+$, Her2$^+$, and the highly aggressive TNBCs (Fig. 1B–D). The LN-positive cases showed poor outcome compared with LN-negative category (Fig. 1E). Within the LN-positive cases also, patients with clNotch^{high}pErk^{high} expression showed poor outcome compared with the "rest" (Fig. 1F). OS was shown to diminish with each additional positive node (26); consistent with this, we observed that within the LN-positive category, the clNotch^{high} pErk^{high} group had high LN positivity (pN2 and pN3) compared with the "rest" category that had low LN positivity (pN1; data not shown). Furthermore, even among the node-negative cases within the clNotch^{high} pErk^{high} category, 8 of 10 patients developed relapse and metastasis and died of the disease within 3 to 5 years of follow-up (Fig. 1G), suggesting that hyperactivation of Notch1 and Ras/MAPK pathways may indicate poor prognosis even in this relatively favorable group. Taken together, these data identify an association between coordinate hyperactivation of Notch1 and Ras/MAPK with poor OS in patients with breast cancer, suggesting that Notch1–Ras/MAPK activation status could serve as novel prognostic markers.

Combined inhibition of Notch1 and Ras/MAPK pathways led to decreased proliferation and survival in breast cancer cells

Work from our laboratory and that of others has demonstrated a functional Notch–Ras cooperation in transformation of breast epithelial cells *in vitro* (11, 20, 21). The present study revealed an association of elevated activation of Notch1 and pErk1/2 with poor prognosis (Fig. 1, Table 1), together suggesting that Notch–Ras activation in breast cancers may contribute to breast cancer development and aggressiveness. This led us to investigate the outcome of combinatorial targeting of Notch1 and Ras/MAPK pathways in breast cancers. Many studies that aimed at targeting Notch signaling have used γ-secretase inhibitors (GSI; refs. 27, 28). However, GSIs target more than 30 physiologically important transmembrane proteins (29), and clinical trials of GSIs have been hindered by considerable gastrointestinal problems experienced by the participants (30). As an alternative, selective blocking of Notch1 receptor with antibody has been shown to inhibit cancer cell growth in preclinical models (31). Accordingly, we used an in-house–generated anti-Notch1 mAb, MAb602.101, whose effectiveness in targeting Notch1 signaling in breast cancer cells was recently reported by us (15). Treatment with MAb602.101 also led to a reduction in the expression of downstream targets of Notch signaling, such as, Hes1, Hes5, HeyL, and uPA (Supplementary Fig. S2A). To inhibit the Ras/MAPK pathway, we used PD98059 that inhibits MEK1 (MAPK/Erk kinase-1), thus preventing phosphorylation and activation of Erk1/2 (32). Treatment of BT474 breast cancer cells with PD98059 led to a reduction in the levels of pErk (Supplementary Fig. S2B). In addition, we noticed that treatment with PD98059 also led to a reduction in the levels of Jagged1, and a few of the Notch pathway downstream targets (Supplementary Fig. S2C), consistent with the regulation of the Notch pathway by the Ras/MAPK pathway (33). Combined treatment with MAb602.101 and PD98059 led to a stronger inhibition of the Notch pathway (Supplementary Fig. S2D). Similar results were obtained in yet another breast cancer cell line MDA-MB-231 (data not shown).

We first treated different types of breast cancer cells BT-474 (ER$^+$, PR$^-$, and Her2$^+$), MCF-7 (ER$^+$, PR$^-$, and Her2$^-$), MDA-MB-231 (ER$^-$, PR$^-$, and Her2$^-$), and HCC-1806 (ER$^-$, PR$^-$, and Her2$^-$) with MAb602.101 and PD98059, alone or together, in a dose-dependent manner, and evaluated the effects on proliferation and survival *in vitro*.

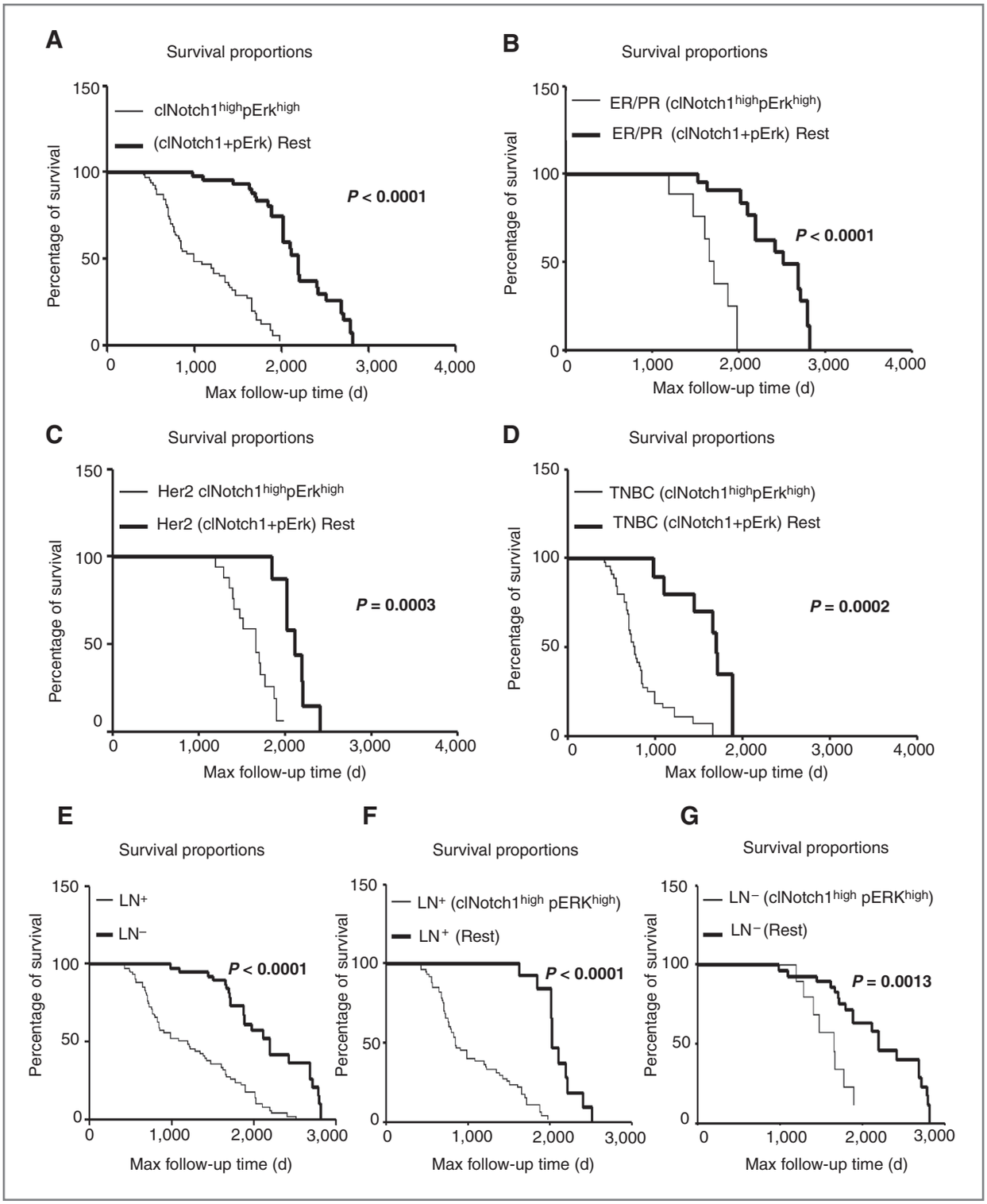

Figure 1. Correlation of Notch1 and Ras/MAPK pathway status with patient survival. Graphs, Kaplan–Meier analysis of clNotch1^{high}pErk^{high} versus "rest" subsets for total of 115 patients with breast cancer (A), ER/PR^{+} (B), Her2^{+} (C), patients with TNBC (D), and LN status (E–G).

Our results demonstrated that compared with individual inhibition, whereas combinatorial inhibition of Notch1 and Ras/MAPK pathways led to a slight reduction in proliferation (Fig. 2A–C) and viability (Supplementary Fig. S3A–S3C), it led to a significant increase in apoptotic cell death of these cells (Fig. 2D and E). These results

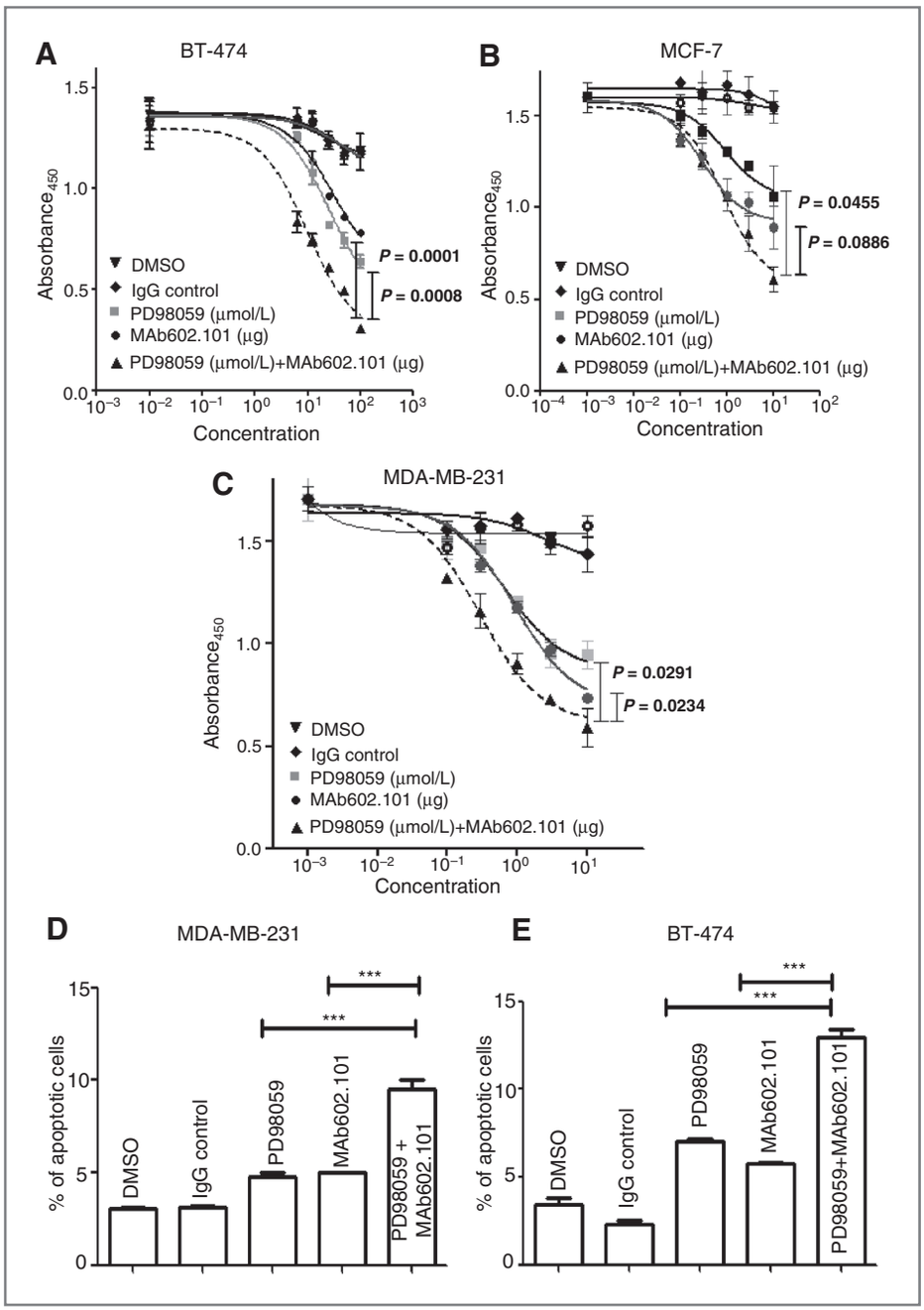

Figure 2. Effect of Notch1 and Ras/MAPK inhibition on proliferation and apoptosis in breast cancer cell lines. A–C, graphs, proliferation (as assessed by BrdUrd incorporation) of breast cancer cell lines BT-474 (A), MCF-7 (B), and MDA-MB-231 (C) following 72 hours of treatment with anti-Notch1 MAb 602.101 (10 μg/mL) and MEK inhibitor PD98059 (10 μmol/L), alone or together; treatment with IgG and DMSO served as controls. D and E, bar graphs, apoptotic cell death in MDA-MB-231 (D) and BT-474 (E) cells following 72 hours of treatment with anti-Notch1 MAb 602.101 (10 μg/mL) and MEK inhibitor PD98059 (10 μmol/L), alone or together; treatment with IgG and DMSO served as controls. Cells were stained with Annexin V–PE-Cy5 and analyzed by flow cytometry for assessing apoptotic cell death; results, means ± SD; $n = 3$. ***, $P < 0.001$.

indicated that concurrent inhibition of Notch1 and Ras/MAPK pathways might provide an effective treatment approach against multiple subtypes of breast cancers, including the highly aggressive TNBCs.

Combinatorial inhibition of Notch1 and Ras/MAPK inhibits sphere formation and reduces the CD44^{high}/CD24^{low}subpopulation of breast cancer cells

Recent studies have identified stem-like cancer cells within several cancers (34). Because Notch1 signaling is implicated in the regulation of self-renewal (14, 15), and the Ras/MAPK pathway is associated with proliferation and survival, we investigated the effectiveness of combinatorial inhibition of Notch1 and Ras/MAPK pathways in the maintenance of breast cancer stem-like cells, as compared with individual inhibition of these two pathways. The ability to generate anchorage-independent, 3-dimensional spheroids *in vitro* serves as a measure of putative self-renewing stem-like cells in mammary cells (14, 15). Accordingly, we investigated the effects of combinatorial inhibition of Notch1 and Ras/MAPK pathways on sphere-

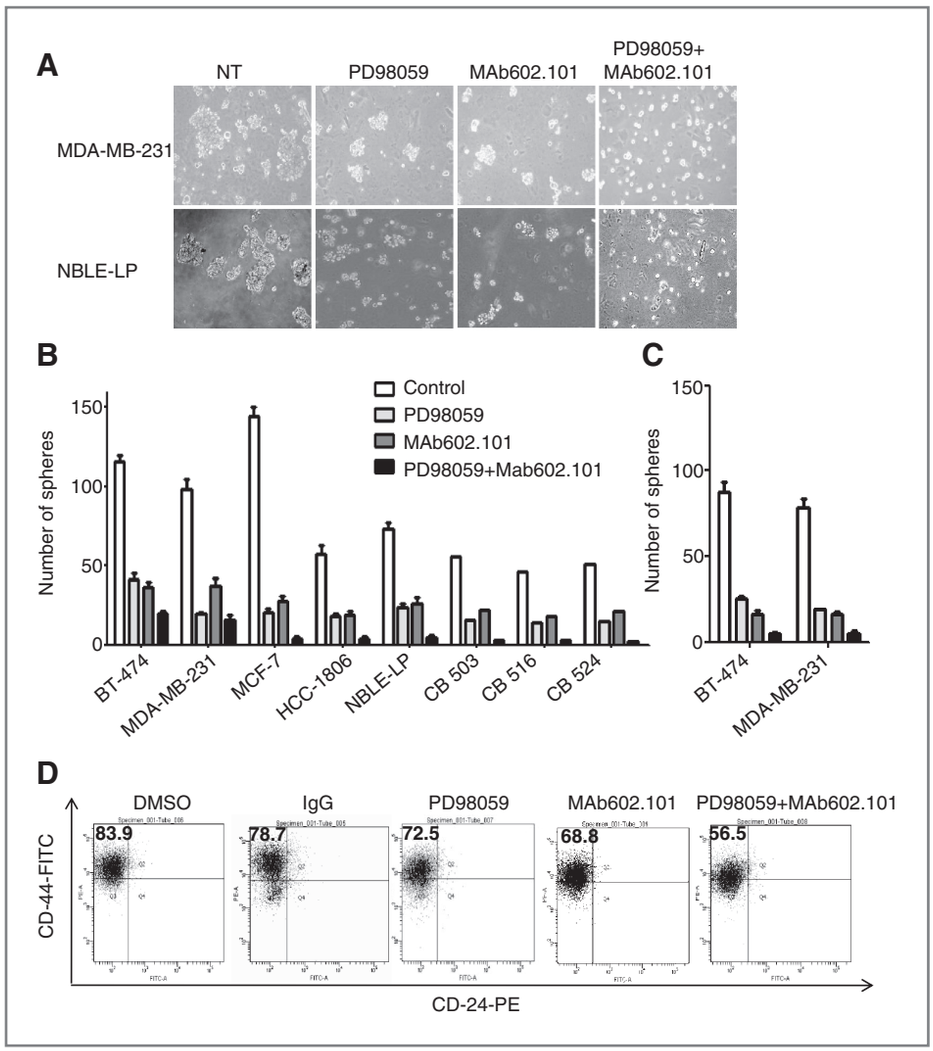

Figure 3. Effect of Notch1 and Ras/MAPK inhibition on mammosphere formation and $CD44^{high}/CD24^{low}$ subpopulation in breast cancer cell lines. A and B, breast cancer cell lines were assayed for sphere formation in the presence of anti-Notch1 MAb602.101 (10 μg/mL), 10 μmol/L MAPK inhibitor (PD98059), combination of MAb602.101 and PD98059, and appropriate controls (IgG and DMSO) for 1 week. A, photomicrographs represent phase contrast images of spheres formed by MDA-MB-231 and NBLE-LP cells; magnification, ×10. B, quantification of the sphere-forming capacity of BT-474, HCC-1806, MDA-MB-231, MCF-7, NBLE-LP, and three primary breast cancer tissues (one $Her2^+$ case and two TNBC cases), in the same assay. C, after 1 week of treatment as above, BT474 and MDA-MB-231 cells from experiment (B) were replated for secondary sphere formation in the absence of inhibitors. Graphs, quantification of secondary spheres formed. D, histograms show flow-cytometry analyses for the expression of cell surface markers CD44 and CD24 in MDA-MB-231 cells treated with anti-Notch1 MAb (602.101), MAPK inhibitor (PD98059), combination of MAb602.101 and PD98059, and appropriate controls (IgG and DMSO) for 72 hours; results, means ± SD; $n = 3$. The experiments were repeated three times.

forming potential. Although individual treatments did show a decrease in the number and sizes of spheres formed compared with controls, interestingly, combinatorial inhibition of Notch1 and Ras/MAPK pathways led to a marked abrogation of sphere formation in all the cell lines analyzed (Fig. 3A and B). Furthermore, when treated spheres were disaggregated and replated in the absence of inhibitors, we noticed that although the combinatorial inhibition led to complete inhibition of secondary sphere formation, the individual treatments led to the formation of fewer and smaller sized spheres (Fig. 3C and Supplementary Fig. S4A). This is consistent with our previous data in which we demonstrated that anti-Notch1 antibodies deplete breast cancer stem-like cells and irreversibly affect sphere-forming potential of breast cancer cell lines (15).

Recently, we reported the generation of NBLE cells by *in vitro* transformation of normal mammospheres (22); later passages of this cell line (NBLE-LP) showed enhanced sphere-forming potential, and comprised of greater than 90% of cells showing $CD44^{high}/CD24^{low/-}$ phenotype, which identifies the breast cancer stem cells (35). Interestingly, combinatorial inhibition of Notch1 and Ras/MAPK abrogated sphere formation in these stem cell–enriched cells also (Fig. 3A and B). To further validate these results, we used patient-derived cancer mammospheres. Combinatorial inhibition led to complete abrogation of sphere formation in all three primary patient samples tested (Fig. 3B). Furthermore, combinatorial inhibition of the Notch1 and Ras/MAPK pathways also led to a significant reduction in the $CD44^{high}/24^{low/-}$ subpopulation compared with individual targeting (Fig. 3D). Together, these data indicated that combinatorial inhibition of Notch1 and Ras/MAPK effectively abrogates sphere formation and reduces the $CD44^{high}/24^{low/-}$ putative breast cancer stem-like cells.

Combinatorial inhibition of Notch1 and Ras/MAPK causes tumor regression *in vivo*

To further investigate the potential efficacy of combining Notch1 and Ras/MAPK inhibition, we performed

preclinical xenograft tumor assays. The breast cancer cell lines BT-474, MDA-MB-231, and HCC-1806 were grown as xenografts in nude mice until they reached the size of 100 mm^3. The mice were treated with PD98059 (intratumorally) and MAb602.101 (i.p.), alone or together. In all the xenograft models, although individual inhibition of Notch1 or Ras/MAPK pathways led to slight retardation of tumor growth, combinatorial inhibition of these two pathways almost completely impeded tumor growth (Fig. 4A–D and Supplementary Fig. S5). These results highlight the importance of combinatorial inhibition of Notch1 and Ras/MAPK pathways in targeting breast cancers. Furthermore, because combinatorial treatment impeded tumor formation in TNBC cell lines (MDA-MB-231 and HCC-1806), our results additionally reveal a novel treatment strategy to target this highly aggressive cancer subtype that currently lacks targeted treatment options.

Discussion

Hyperactivation of the Notch1–Ras/MAPK pathway as prognostic markers in breast cancer

The TNM (tumor size, node, and metastasis) staging system has been the classical and most widely used system to provide prognostic information regarding a patient. Besides TNM, standard predictive markers for breast cancer treatment include hormone receptor expression for endocrine therapy and HER2 status for anti-HER2 therapy (4). There is a further need for better prognostic and predictive markers that can enable improved categorization of breast cancers, which can in turn help the correct choice of treatment. With the launch of high-throughput technologies in recent years, a number of multigene signatures have been identified (36, 37) that, together with the traditional markers, can serve as better prognostic and predictive markers. Increased Notch receptors, ligands, and consequent increase in Notch activity have been reported in breast cancers (11, 38). Coexpression of Notch1 and Jag1 has been associated with poor prognosis (10). In this investigation, we show that a large number of patients with grade 3 invasive ductal carcinoma of the breast expressed high levels of cleaved Notch1 and pErk1/2, suggestive of coordinate hyperactivation of the Notch and Ras/MAPK pathways. In patients who presented with high levels of cleaved Notch1 and pErk1/2, we observed an early relapse to vital organs like brain, liver, and lungs. This was consistent with poor survival with a median survival of 982 days in patients displaying a $clNotch^{high}pErk^{high}$ phenotype compared with 2,705 days in those exhibiting low or no expression of these proteins. Thus, these results suggested that coexpression of cleaved Notch1 and pErk1/2 might act as new markers for better stratification and predicting the prognostic behavior of patients with breast cancer.

Furthermore, our study shows for the first time that a large number of TNBCs (81%), displaying the expression of stemness markers like Oct4, nanog, and CD44, also showed increased coordinate activation of Notch1 and Ras/MAPK pathways. TNBCs are associated with a shorter median time to relapse and death; therefore, one chief objective is the identification of prognostic factors and markers to efficiently select high- and low-risk subsets of patients with TNBC for different treatment regimens. Our survival analyses revealed that the patients with TNBC that fall into $clNotch^{high}pErk^{high}$ category have much poorer survival, suggesting that hyperactivation of Notch1 and Ras/MAPK pathways may be used as predictive markers for this aggressive group of breast cancers.

Combinatorial targeting of Notch1 and Ras/MAPK in breast cancer

Various groups have shown aberrant activation of Notch (10, 17) and Ras pathways (39) in breast cancer and proposed their independent inhibition as strategies to target breast cancer (16, 40). Emerging studies though show that combinatorial targeting of multiple pathways in cancer is likely to have a better therapeutic effect than solitary approaches (18). Several lines of evidence indicate that Notch inhibitors may prove beneficial in combination with these therapies used for ER^+, $Her2^+$, and TNBCs (41). On the basis of our study that revealed association of coordinate hyperactivation of Notch1 and Ras/MAPK pathways with increased risk of node positivity and overall poor outcome, we investigated the effectiveness of combinatorial inhibition of these two pathways in targeting breast cancer. Our results revealed that combinatorial inhibition of Notch1 and Ras/MAPK not only led to effective reduction in proliferation and survival in various breast cancer cell lines tested, but also resulted in increased apoptosis. In sphere formation assays that test for the self-renewing potential of putative cancer stem-like cells, while individual treatments led to a reduction in sphere number and size, importantly, combinatorial treatment completely abrogated sphere formation. Furthermore, combined inhibition of Notch1 and Ras/MAPK led to a significant decrease in the $CD44^{high}/CD24^{low/-}$ cells that represent the sphere-forming, chemotherapy-resistant cancer stem-like cells in breast cancers (35). These results indicated that combinatorial inhibition of Notch1 and Ras/MAPK pathways may offer therapeutic opportunity for breast cancers, at least in part, by targeting the cancer stem-like cells.

Recently, Liu and colleagues (42) provided mathematical modeling supporting the idea of combinatorial therapy to target cancer progression. Consistent with this, we show that although individual inhibition of Notch1 and Ras/MAPK pathways led to a reduction in tumor growth of BT-474, MDA-MB-231, and HCC-1806 cells in tumor xenograft assays, combinatorial inhibition of these two pathways completely impeded the growth of these cells *in vivo*, thus demonstrating the better efficacy of combinatorial inhibition of these two pathways in treating breast cancers. Furthermore, Notch and Ras activation have both been implicated in the development of therapy resistance in standard breast cancer treatments (43). Thus, our data

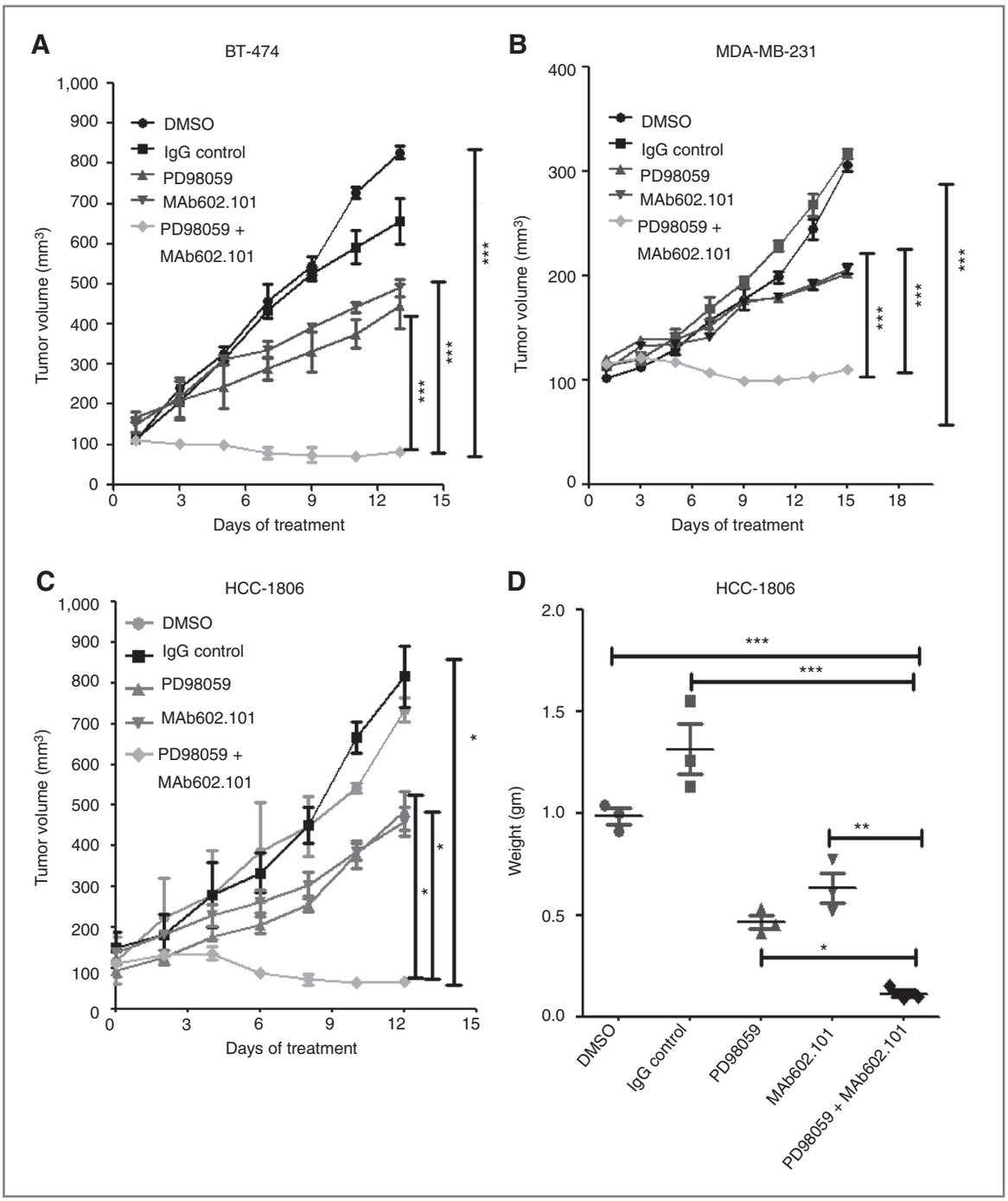

Figure 4. Effect of Notch1 and Ras/MAPK inhibition on *in vivo* tumor growth. A, BT-474; B, MDA-MB-231; and C and D, HCC-1806 (1×10^6) were injected s.c. into nude mice and allowed to attain a volume of 100 to 200 mm^3. Animals were then administered with intratumoral injections of DMSO or PD98059 (50 μmol/L), i.p. injections of control IgG or MAb602.101 (15 mg/kg b.w.) and combination of MAb602.101 and PD98059 every 48 hours and the tumor volume was determined every third day and plotted graphically; results, means ± SD; $n = 6$. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

additionally provide crucial experimental insights into the feasibility of a combinatorial strategy to overcome therapy resistance in breast cancers.

Standard cytotoxic chemotherapy is the method of choice to treat TNBCs, which often results in disease relapse. In the last few years, various signal transduction

pathways have been proposed to play a role in regulating growth and survival in developing chemoresistance in TNBC (44). Recently, it was shown that dual inhibition of Met and Notch may prove beneficial for patients with TNBC with Met overexpression and Notch hyperactivation (45). On the basis of our data revealing a strong association of Notch1 and Ras/MAPK pathway activation in TNBCs, and the good response of TNBC cell line–derived tumors in preclinical testing with combinatorial inhibition of these two pathways, we propose combinatorial inhibition of Notch1 and Ras/MAPK pathways as novel therapeutic strategies for treating TNBCs, which currently lack such targeted therapeutic modules.

Several clinical studies focusing on the inhibition of Notch and Ras/MAPK pathways have been undertaken and several others are underway (http://www.cancer.gov/clinicaltrials; refs. 16, 19, 27, 28, 46). One major approach that is being tried clinically for Notch inhibition includes the use of small-molecule GSIs that block the proteolytic activation of Notch (27, 46). A phase I clinical study with GSI MK-0752 (Merck) reported adverse gastrointestinal effects. Using several dosing schedules, another GSI RO4929097 was reported to show a tolerable safety profile and some antitumor activity. An alternate approach for modulating Notch signaling involves the use of blocking mAbs targeting various Notch receptors and ligands. Currently, a dose-escalation phase I clinical trial of OMP-59R5, a humanized mAb that blocks Notch 2 and 3 signaling, and other clinical trials using OMP-21M18, humanized mAb antibody against Notch ligand DLL4, in different solid tumors are ongoing (27, 46). Similarly, several MEK 1/2 inhibitors such as like AZD8330 (Array BioPharma/AstraZeneca), RO5126766, and RO4987655 (Hoffmann La Roche) are being evaluated in phase I clinical trials of patients with advanced cancer (19, 47), whereas the FDA-approved MEK inhibitor, trametinib (Mekinist) is under use for the treatment of patients with advanced melanoma. Many trials combine GSIs with other agents, including tyrosine kinase inhibitors, mammalian target of rapamycin inhibitors, aromatase inhibitors, and conventional chemotherapeutics. On the basis of our study, we propose the combinatorial inhibition of Notch signaling and Ras/MAPK pathways as a therapeutic approach for breast cancers.

In summary, our study highlights the importance of Notch–Ras cooperation in the pathogenesis of breast cancers and identifies coordinate hyperactivation of the Notch1 and Ras/MAPK pathways as biomarkers for poor prognosis in patients with breast cancer. In addition, our study demonstrates the effectiveness of combinatorial targeting of these two pathways in effectively targeting breast cancers, including the therapy-resistant TNBCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed .

Authors' Contributions

Conception and design: S. Mittal, A. Sharma, S.A. Balaji, A. Rangarajan
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Mittal, S.A. Balaji, A. Rangarajan
Writing, review, and/or revision of the manuscript: S. Mittal, A. Sharma, R.V. Kumar, A. Rangarajan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.C. Gowda, A. Rangarajan
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