High-level Coexpression of JAG1 and NOTCH1 Is Observed in Human Breast Cancer and Is Associated with Poor Overall Survival

Michael Reedijk,[1,2,3] Silvia Odorcic,[1] Lynn Chang,[1] Hui Zhang,[1] Naomi Miller,[4] David R. McCready,[3] Gina Lockwood,[5] and Sean E. Egan[1,6]

[1]Programs in Developmental Biology and Cancer Research, The Hospital for Sick Children; [2]Department of Surgery, Mount Sinai Hospital; Departments of [3]Surgical Oncology, [4]Pathology, and [5]Clinical Study Coordination and Biostatistics, Princess Margaret Hospital, University Health Network; and [6]Department of Molecular and Medical Genetics, The Faculty of Medicine, The University of Toronto, Toronto, Ontario, Canada

Abstract

Aberrant activation of Notch receptors has been shown to cause mammary tumors in mice. We therefore used *in situ* hybridization to analyze expression of Notch ligands and receptors in human breast cancer. High levels of JAG1 and NOTCH1 were noted in a subset of tumors with poor prognosis pathologic features ($P < 0.05$). We therefore used tissue microarrays to analyze the expression of these genes in a collection of breast cancers from patients representing a wide spectrum of clinical stages, and from whom associated follow-up survival data was available ($n = 184$). Patients with tumors expressing high levels of JAG1 or NOTCH1 had a significantly poorer overall survival compared with patients expressing low levels of these genes [5-year survival rate of 42% versus 65% and median survival of 50 versus 83 months, respectively, for $JAG1^{Hi\ vs.\ Lo}$ ($P = 0.01$); 49% versus 64% and 53 versus 91 months, respectively, for $NOTCH1^{Hi\ vs.\ Lo}$ ($P = 0.02$)]. Moreover, a synergistic effect of high-level JAG1 and high-level NOTCH1 coexpression on overall survival was observed (5-year survival rate of 32% and median survival of 40 months; $P = 0.003$). These data (*a*) identify novel prognostic markers for breast cancer, (*b*) suggest a mechanism whereby Notch is activated in aggressive breast tumors, and (*c*) may identify a signaling pathway activated in poor prognosis breast cancer which can be therapeutically targeted. (Cancer Res 2005; 65(18): 8530-37)

Introduction

Breast cancer is the most commonly diagnosed malignancy, and is a leading cause of cancer death in females from western countries (1). A continued focus on elucidating the molecular mechanisms underlying this disease is necessary to improve on current limited treatment success. Mutations in human breast cancer have been identified that activate expression or elevate the function of oncogenes (2), and that disrupt tumor suppressor genes (3). In some cases, specific molecular lesions have been associated with poor prognosis (4, 5) or with specific breast tumor types (6). More recently, microarray studies have helped to define gene expression profiles that correlate with specific breast tumor subtypes and with patient outcome (7–9). This work has defined "Basal" and "ErbB2-positive" subtypes as particularly aggressive, and has led to the suggestion that transformation of the most primitive mammary stem/progenitor cell may give rise to tumors which express basal or myoepithelial cell markers (10).

Notch receptors are large transmembrane epidermal growth factor–like repeat-containing proteins that regulate a number of cellular properties important in cancer, including cell division, differentiation, and survival (11–13). In addition, Notch signaling has been implicated in self-renewal of stem cells, including stem, and/or progenitor cells isolated from the mammary gland (14, 15). Mammals have four Notch receptors: NOTCH1, 2, 3, and 4 (Fig. 1). These receptors are activated in most contexts by mammalian Delta (Delta-like) and Serrate (Jagged) ligands, which are also transmembrane proteins containing multiple epidermal growth factor-like repeats (known as DSL ligands). Once activated, Notch receptors are cleaved by the γ-secretase protease complex to release a cytoplasmic domain fragment, $Notch^{IC}$, that translocates into the nucleus (16, 17). Once in the nucleus, $Notch^{IC}$ binds to the conserved DNA-binding protein, RBPJκ/CBF-1, as well as to Mastermind and other transcriptional regulatory proteins to form a complex which induces expression of genes involved in cell growth, differentiation, and cell survival (16–19). Although less characterized, Notch receptors can also directly activate signal transduction pathways in the cytoplasm.

In the early 1990s, Callahan and colleagues identified Notch4 as a potent oncogene, selected for in mouse mammary tumor virus–induced mammary tumors (20, 21). In fact, activated Notch4 was able to transform mammary epithelium *in vitro* (20) and *in vivo* (22–24). Interestingly, an activated *Notch4* oncogene (*Int3*) slowed ductal growth and perturbed lobular outgrowth prior to induction of tumor formation (23). Activated Notch4 had the opposite effect to Wnt signaling on TAC-2 mammary epithelial cell branching *in vitro* (25), suggesting that these two pathways transform cells through very distinct mechanisms. Activated Notch1 can also transform mammary epithelium (26, 27). Here we report on expression of Notch ligands and receptors in human breast tumors. We have determined that JAG1, a ligand that is normally expressed in basal epithelial cells of the mammary gland, is highly expressed in a population of human breast tumors, and that patients with these tumors show dramatically reduced overall survival. We also note that high-level coexpression of JAG1 and NOTCH1 is associated with a further reduction in overall survival.

Materials and Methods

Breast tumor tissue. Human breast cancer specimens from tumors >2.0 cm in diameter (Supplementary Table S1) were obtained through a written

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Sean E. Egan, Program in Developmental Biology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G-1X8. Phone: 416-813-5267; Fax: 416-813-8883; E-mail: segan@sickkids.ca.

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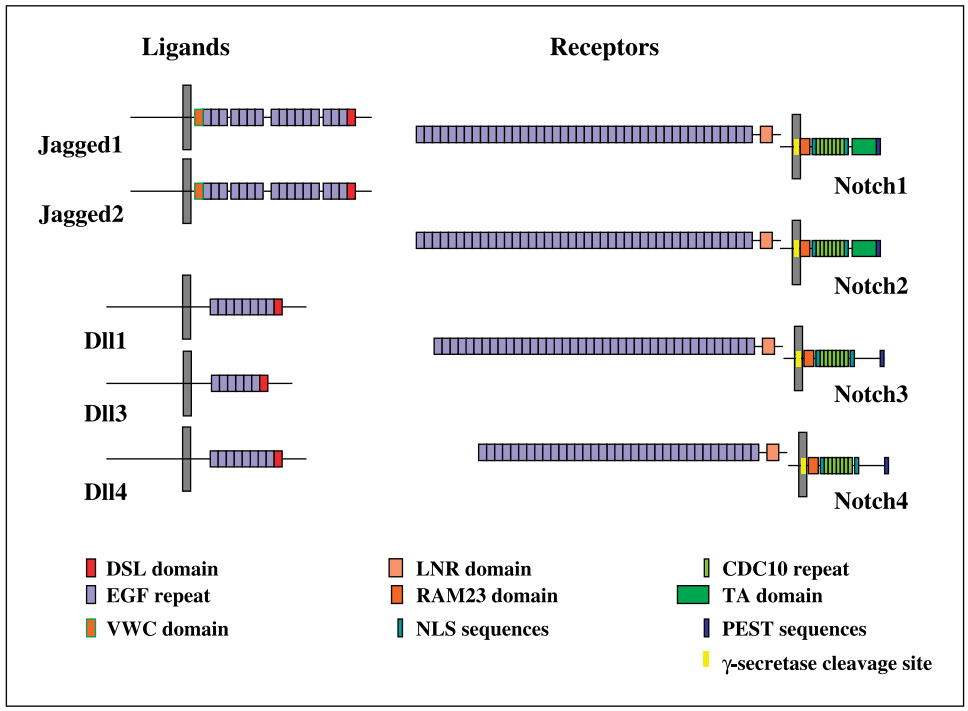

Figure 1. Notch ligands and receptors. Schematic diagram of DSL Notch ligands (Jagged1,2, Delta1,3,4) and Notch receptors (1-4). Both ligands and receptors contain multiple conserved domains identified by colored boxes.

informed consent process that adhered to stringent ethical criteria from the University Health Network of the University of Toronto. Within 5 to 15 minutes of resection, tumor specimens were placed in an RNase-free solution of 4% paraformaldehyde in PBS (pH 7.4) and incubated at room temperature overnight to allow fixation. The tissues were then washed for 30 minutes in each of PBS, saline, saline/ethanol (1:1), and 70% ethanol in saline. Paraffin blocks were generated in the standard fashion. Breast cancer tissue microarray slides were provided by the Cooperative Breast Cancer Tissue Resource, which is funded by the National Cancer Institute. These tissue microarrays included 192 0.6 mm samples of invasive primary ductal breast cancer (64 cases each of node-negative, node-positive, and metastatic breast cancer; Supplementary Table S2).

Generation of probes for *in situ* hybridization. cDNAs encoding Notch ligands and receptors were obtained from the IMAGE consortium. Fragments of these (Supplementary Table S5) were subcloned into expression vectors using standard techniques. Antisense ^{33}P-UTP-radiolabeled cRNA probes for *in situ* hybridization were generated using T3 or T7 RNA polymerase as per standard procedures.

***In situ* hybridization and immunohistochemistry.** Tumor tissue sections were dewaxed in xylene and hydrated by serial incubations in decreasing concentrations of ethanol made in saline. Tissues were refixed in 4% paraformaldehyde/PBS, washed in PBS, and treated with 20 μg/mL of proteinase K (Invitrogen, Carlsbad, CA) for 7.5 minutes. This was followed by a wash in PBS, another fixation in 4% paraformaldehyde/PBS, and a final PBS wash. To prevent nonspecific binding of probe, tissues were twice acetylated for 5 minutes in 0.1 mol/L triethanolamine-HCl containing 500 μL of acetic anhydride and 448 μL of 10 N NaOH. After 5-minute incubations in PBS followed by saline, tissues were dehydrated through a stepwise process and air-dried. Radiolabeled probe was placed in hybridization mixture (50% deionized formamide, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 10 mmol/L $NaPO_4$, pH 8.0, 10% dextran sulfate, 1× Denhardt's solution, 0.5 mg/mL yeast tRNA, and 10 mmol/L DTT) to a final concentration of 1.5×10^5 cpm/μL and denatured at 80°C for 2 minutes. Prepared tissue sections were covered with 60 μL of probe/hybridization mixture under a coverslip and allowed to incubate in a sealed container ON at 55°C. Coverslips were removed with a brief incubation in 5× SSC/0.1% 2-mercaptoethanol at 55°C. Slides were then placed in 50% formamide/2× SSC/2-mercaptoethanol at 65°C for 30 minutes. Next, slides were washed thrice in 0.5 mol/L NaCl/10 mmol/L Tris-HCl/5 mmol/L EDTA at 37°C followed by incubation in 20 μg/mL RNase A (Roche, Indianapolis, IN) for 30 minutes in the same buffer. After a final wash in NaCl/Tris-HCl/EDTA buffer, a repeat high-stringency incubation in formamide/2× SSC/2-mercaptoethanol was done for 30 minutes. Slides were then washed in 2× SSC/2-mercaptoethanol followed by 0.1× SSC/2-mercaptoethanol for 30 minutes each at 65°C. Finally, the tissue sections were dehydrated as described above. Slides were treated with Kodak NTB-2 nuclear emulsion and stored at 4°C for ~3 weeks prior to development. Slides were developed in Kodak D-19 solution, fixed in Kodafix and counterstained with 0.1% toluidine blue. Immunostaining for cytokeratin 5 and cytokeratin 17 was performed as described (28).

Quantification. In RNA *in situ* hybridization, a linear relationship exists between the level of radioactivity hybridized to a tissue specimen and the concentration of activated silver grains (determined either by an image

Table 1. Patient characteristics

	University Health Network	National Cancer Institute
n	51	184
Age*	59 (29-86)	59 (29-89)
Tumor size (cm)*	3.7 (1.2-18)	2.3 (0.1-6)
Grade (%)		
1	12	17
2	25	64
3	63	18
Node-positive (%)	57	59
Necrosis (%)	34	—
LVI (%)	49	—
ER-positive (%)	67	65
PR-positive (%)	45	33
c-ERB2/neu–positive (%)	19	—

*Mean (range).

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Table 2. Expression of Notch ligand and receptor mRNA in human breast cancers from the University Health Network

mRNA probe	Tumor
DLL1	2^{Hi} of 22
DLL3	0 of 22
DLL4	0 of 22
JAG1	6^{Hi} of 47
JAG2	9^{Hi} of 22
NOTCH1	5^{HI} of 39
NOTCH2	19^{Hi} of 22
NOTCH3	6^{Hi} of 50
NOTCH4	1^{Hi} of 22

analyzer or by manual counting; ref. 29). For tissue sections generated from the University Health Network tumors, quantitation of mRNA expression was done through manual counting of silver grains. For NIH tissue microarrays, dark-field 20× magnification views of tumor tissue sections were digitally photographed (8-bit gray scale). Exposure time was standardized for all photographs. Using Image-Pro Plus (Media Cybernetics, Inc., San Diego, CA) image analysis software, the concentration of activated silver grains was determined at four locations over the tumor and an average was obtained. As tumors could not easily be separated into distinguishable groups on the basis of distinct expression levels, we arbitrarily defined high expressing tumors (Hi) for each probe as those expressing a gene in the highest quartile of the expression range in each case. Expression levels between different mRNA targets could not be compared with this method because of inherent differences in binding kinetics for each probe.

Statistical analysis. Predicted 10-year risks of mortality and relapse were calculated using Adjuvant! for each University Health Network breast cancer specimen for which all requested pathologic variables were available. Means, SDs, and medians were calculated for the specimens with high expression of JAG1, NOTCH1, or NOTCH3 and specimens with low expression. These groups were compared using Mann-Whitney tests. For the 192 invasive primary ductal breast cancer samples obtained from the National Cancer Institute, overall survival was measured from diagnosis to death or last follow-up. Kaplan-Meier curves were calculated for the high and low expression JAG1, NOTCH1, and NOTCH3 groups. Survival between groups was compared using the log-rank test. The coexpression of high levels of JAG1 and NOTCH1 was similarly investigated. Cox proportional hazard regression was used to look for a dose-response relationship between level of gene expression and survival. Bivariate models examined whether gene expression had an independent effect on survival after controlling for known predictors. Coexpression of high levels of JAG1 and NOTCH1, JAG1 and NOTCH3, and NOTCH1 and NOTCH3 were examined in contingency tables and tested for independence using the χ^2 test. P values ≤ 0.05 were considered statistically significant.

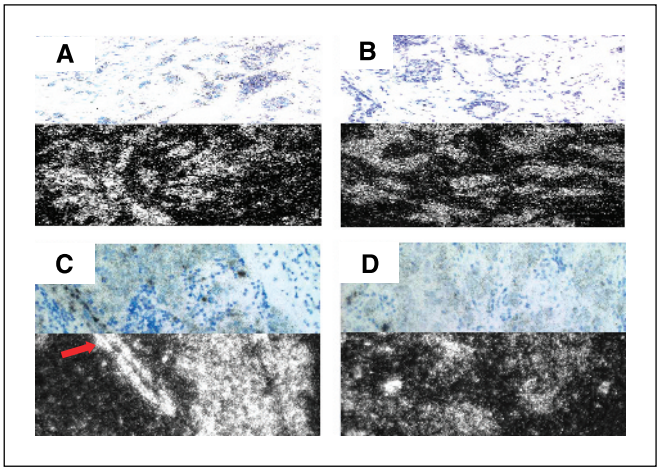

Figure 2. Expression of Notch receptors and ligands in breast cancer. *In situ* hybridization using NOTCH1 (*A*), NOTCH2 (*B*), NOTCH3 (*C*), and JAG1 (*D*) antisense riboprobes. High-level Notch receptor and ligand mRNA expression occurs in some invasive breast carcinomas. Photomicrographs are shown at 10× magnification using either bright-field (*top*) or dark-field (*bottom*) microscopy. Solid arrow identifies NOTCH3-expressing vascular smooth muscle cells (*C*).

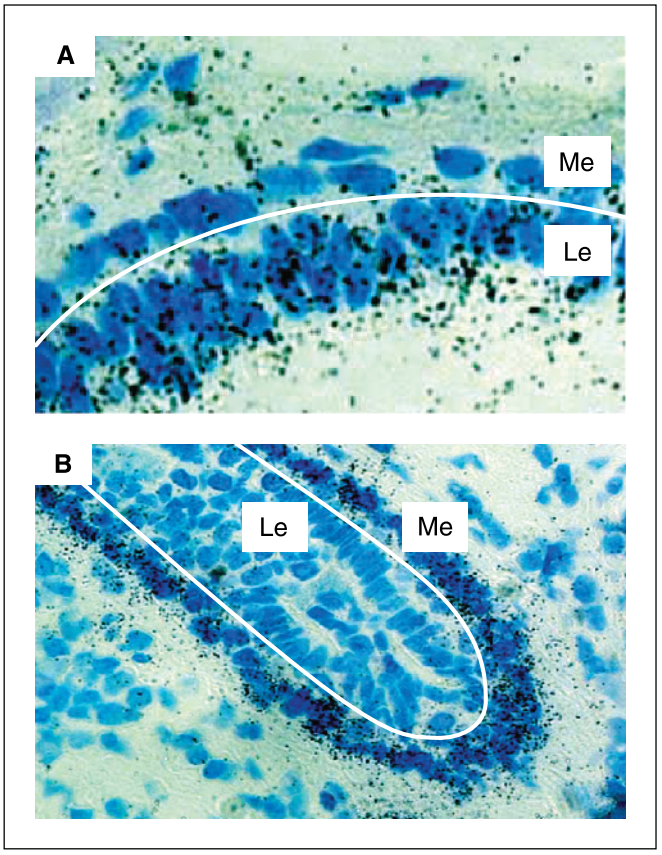

Figure 3. Expression of NOTCH3 and JAG1 in normal mammary epithelium. *In situ* hybridization using NOTCH3 (*A*) and JAG1 (*B*) antisense riboprobes. *A,* NOTCH3 mRNA expression (as identified by activated silver grains) is seen in normal duct luminal epithelium (Le). *B,* normal duct myoepithelium (Me) is positive for JAG1 expression. Photomicrographs are shown at 100× magnification using bright-field microscopy (*A* and *B*).

Results

Analysis of Notch ligands and receptor gene expression in breast cancer. To directly test for expression of Notch ligands (JAG1, JAG2, DLL1, DLL3, and DLL4) and receptors (NOTCH1, 2, 3, and 4) in human breast cancer, as well as in associated blood vessels and stroma, we used mRNA *in situ* hybridization. Our initial screen was done on tumors >2 cm in diameter obtained from patients at the University Health Network (Toronto, Ontario; Table 1). Within this cohort, we identified a group of tumors that coexpressed high levels of Notch ligand, Notch receptor and, in some cases, Fringe gene mRNA (M. Reedijk, et al., in preparation). Therefore, we analyzed Notch ligand and receptor gene expression in a group of up to 50 tumors, and tested for correlations between expression and pathologic data. The DLL1, JAG1, and JAG2 ligands

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Table 3. Adjuvant analysis of breast cancer patients from the University Health Network

		n	Mean (SD)	Median	*P**
Mortality					
JAG1	low	38	7.6 (22.2)	32	0.04
	high	6	55 (15.9)	63	
NOTCH1	low	32	34.9 (21.1)	30.5	0.005
	high	5	70.2 (14.1)	66	
NOTCH3	low	40	37.8 (22.0)	31.5	0.02
	high	6	53.8 (13.8)	55.5	
Relapse					
JAG1	low	38	55.0 (19.7)	51	0.06
	high	6	68.0 (9.7)	71.5	
NOTCH1	low	32	52.7 (18.4)	52	0.004
	high	5	79.8 (11.3)	74	
NOTCH3	low	40	55.6 (19.3)	52	0.09
	high	6	67.5 (10.5)	71	

*P value from Mann-Whitney test comparing two groups.

were expressed at high levels in 2 of 22, 6 of 47, and 9 of 22 tumors, respectively (Table 2; Fig. 2; see Materials and Methods). Notch receptor genes were also expressed at high levels in a variable number of breast tumors: 5 of 39 tumors expressed high levels of NOTCH1, 19 of 22 tumors expressed high levels of NOTCH2, 6 of 50 tumors expressed high levels of NOTCH3, and 1 of 22 tumors expressed high levels of NOTCH4 (Table 2; Fig. 2). Interestingly, many tumors expressed multiple ligands, receptors and Fringes (Supplementary Table S1), however, limitations inherent to *in situ* hybridization prevented us from determining whether these genes were expressed in the same or different cells. In tumor-associated vasculature, a number of ligands and receptors were expressed at high levels (M. Reedijk, et al., in preparation). In addition, some of the tumor samples contained areas of normal mammary tissue, and in these cases, we saw NOTCH3 expression in luminal epithelial cells and JAG1 expression in the surrounding myoepithelial layer, suggesting that this ligand/receptor pair may normally interact in this context (Fig. 3*A* and *B*).

Poor predicted mortality for tumors expressing high levels of JAG1, NOTCH1, or NOTCH3 mRNA. We next tested for an association between Notch ligand or receptor gene expression and 10-year risk of mortality or relapse calculated using Adjuvant!, a widely used clinical tool to predict the risk of negative outcome based on tumor pathologic features and patient characteristics (www.adjuvantonline.com). When tumors were grouped into low

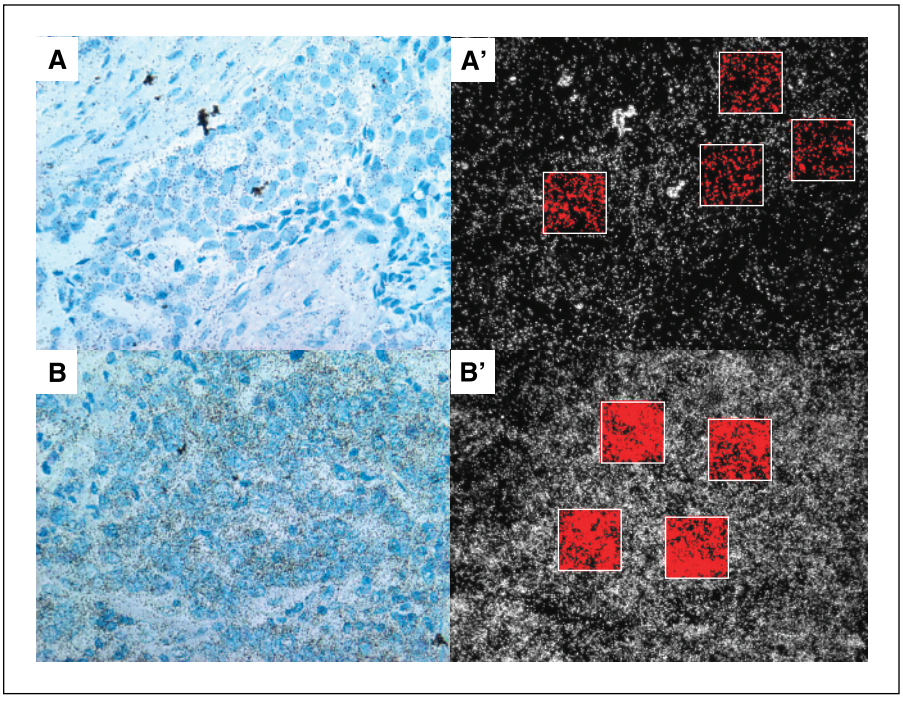

Figure 4. Breast cancers vary in their level of NOTCH1 mRNA expression. Examples of tumors expressing low (*A, A'*) and high (*B, B'*) levels of NOTCH1. Photomicrographs at 20× magnification using either bright-field (*A* and *B*) or dark-field (*A'* and *B'*) microscopy. To quantitate NOTCH1 mRNA expression levels, total silver grain area in four random locations over each tumor specimen was determined (*A'* and *B'*); see Materials and Methods.

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Table 4.

(A) National Cancer Institute survival analysis—Cox proportional hazards model

	n	Hazard ratio (95% CI)	Interquartile range*	Hazard ratio over interquartile range†	*P*‡
JAG1	161	1.11 (1.02-1.21)	3	1.37	0.02
NOTCH1	170	1.06 (0.97-1.15)	3	1.18	0.21
NOTCH3	176	1.06 (0.94-1.20)	1	1.06	0.36

(B) National Cancer Institute survival analysis—comparison of tumors expressing high (Hi) and low (Lo) levels of JAG1, NOTCH1, and NOTCH3 mRNA

		n	5 year survival (95% CI)	Median survival time (95% CI)	*P*§
JAG1	Lo	117	65% (56-74)	83 mo (76-122)	0.01
	Hi	44	42% (27-57)	50 mo (30-78)	
NOTCH1	Lo	126	64% (55-72)	91 mo (72-131)	0.02
	Hi	44	49% (34-64)	53 mo (30-83)	
NOTCH3	Lo	150	61% (53-69)	82 mo (71-122)	0.08
	Hi	26	48% (29-68)	46 mo (23-91)	
$JAG1^{HI}/N1^{Hi}$		22	32% (12-51)	40 mo (19-69)	0.003
$JAG1^{HI}/N1^{Hi}$	excluded	129	63% (54-71)	81 mo (72-103)	
Combined	$J1^{Lo}/N1^{Lo}$	90	62% (51-72)	80 mo (63-131)	
JAG1/N1	$J1^{Lo}/N1^{Hi}$	17	82% (64-100)	84 mo (77-NR)‖	0.02
	$J1^{Hi}/N1^{Lo}$	22	53% (31-74)	71 mo (28-103)	
	$J1^{Hi}/N1^{Hi}$	22	32% (12-51)	40 mo (19-69)	

*Interquartile range = third quartile value − first quartile value.
†Hazard ratio comparing a subject in the highest quarter for the measure to a subject in the lowest quarter.
‡*P* value from Cox proportional hazards model.
§*P* value from log rank test.
‖Upper limit not reached.

expressers and high expressers for each gene, and pathologic data analyzed for predicted mortality and relapse in each group (see Materials and Methods), we observed a statistically significant relationship between high-level JAG1 expression and increased predicted mortality when compared with tumors expressing low levels (median predicted mortality, 63% for $JAG1^{Hi}$ versus 32% for $JAG1^{Lo}$; $P = 0.04$; Table 3). Similarly, we observed statistically significant relationships between high-level NOTCH1 or NOTCH3 expression and increased predicted mortality (at 10 years) when compared with tumors expressing low levels of these receptors (median predicted mortality, 66% for $NOTCH1^{Hi}$ versus 30.5% for $NOTCH1^{Lo}$; $P = 0.005$; and median predicted mortality, 55.5% for $NOTCH3^{Hi}$ versus 31.5% for $NOTCH3^{Lo}$; $P = 0.02$; Table 3). Predicted relapse data showed the same trends, although conventional significance was only reached for NOTCH1 expression (median predicted relapse, 71.5% for $JAG1^{Hi}$ versus 51% for $JAG1^{Lo}$; $P = 0.06$; predicted relapse, 74% for $NOTCH1^{Hi}$ versus 52% for $NOTCH1^{Lo}$; $P = 0.004$; and predicted relapse, 71% for $NOTCH3^{Hi}$ versus 52% for $NOTCH3^{Lo}$; $P = 0.09$; Table 3). We did not observe any other statistically significant relationships between Notch ligand or receptor gene expression and predicted outcomes in this small survey, such as the positive relationship between NOTCH2 expression and patient survival noted in a recent reverse transcription-PCR–based study (30). Interestingly, tumors in this small sample with high levels of JAG1, NOTCH1, or NOTCH3 were almost exclusively ER-negative, PR-negative, and ErbB2-negative but basal cytokeratin protein expression–positive (Supplementary Table S1)—defining characteristics of the basal subtype of breast cancer (7–9).

Elevated JAG1 expression is an independent predictor of poor outcome in breast cancer. Based on these results, we analyzed JAG1, NOTCH1, and NOTCH3 expression in a large panel of breast cancers with associated patient follow-up data. For this study, we obtained tissue microarrays from the U.S. National Cancer Institute Cooperative Breast Cancer Tissue Resource. These tissue microarrays were constructed from a cohort of tumors that were 1/3 node-negative, 1/3 node-positive, and 1/3 metastatic ($n = 64$ for each group; Table 1). Once again, we used *in situ* hybridization to analyze tumor-specific expression. For these experiments, we modified our system for gene expression quantitation by using image analysis software to determine the concentration of activated silver grains in multiple areas of tumor for each sample (see Materials and Methods; Fig. 4). Expression data for JAG1, NOTCH1, and NOTCH3 are tabulated together with data on individual patient and tumor characteristic (Supplementary Table S2).

We first tested for relationships between expression of each gene and overall patient survival. Interestingly, JAG1 expression data showed a dose-dependent relationship with negative outcome (data not shown). For example, patients with tumors expressing JAG1 at levels within the top 25% of the expression range were 37% more likely to die than patients with tumors expressing JAG1 at levels in the bottom quartile expression group (hazard ratio over interquartile range of 1.37; $P = 0.02$;

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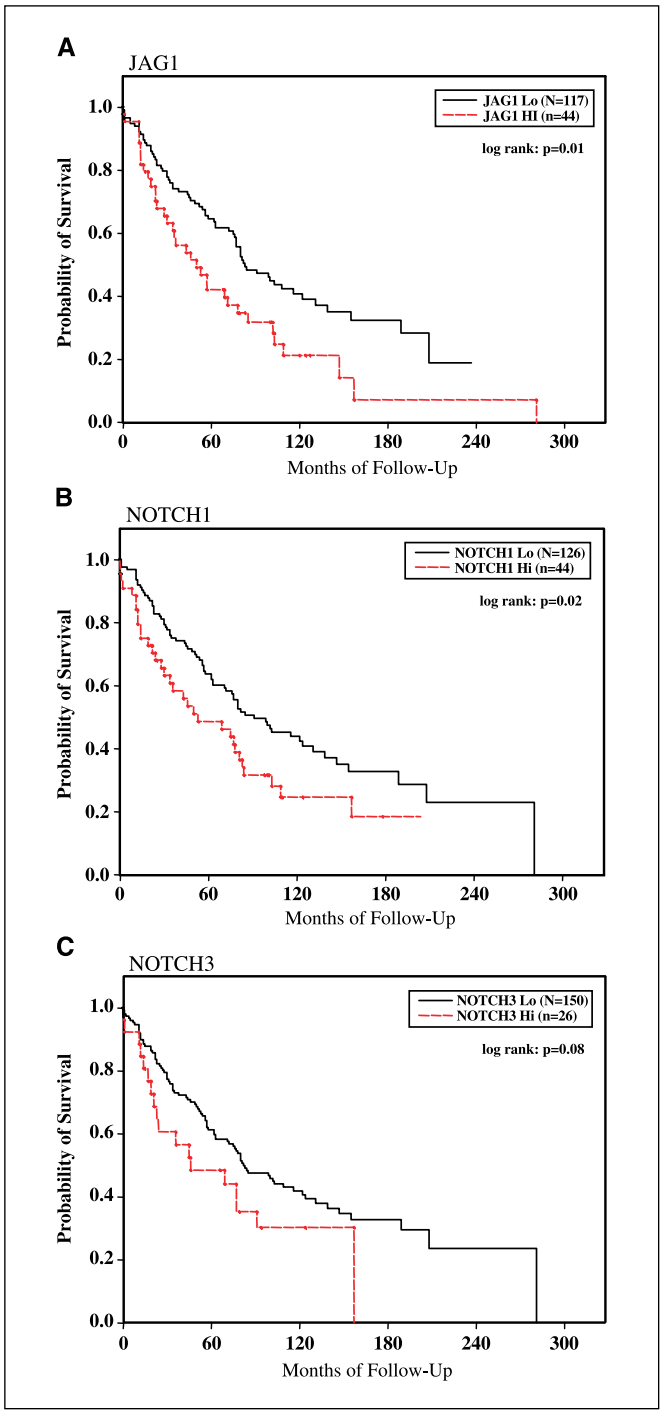

Figure 5. Kaplan-Meier curves showing relationship between high-level JAG1, NOTCH1, or NOTCH3 expression and overall survival in patients with breast cancer. Patients with tumors expressing high levels of JAG1 (*A*), or NOTCH1 (*B*) have significantly shorter survival (JAG1, $P = 0.01$; NOTCH1, $P = 0.02$; log rank test). Patients with tumors expressing high levels of NOTCH3 (*C*) showed a trend ($P = 0.08$) towards shorter survival.

Table 4A). We next compared overall survival in patients whose tumors expressed low levels of JAG1 ($JAG1^{Lo}$) to patients with tumors expressing high levels of JAG1 ($JAG1^{Hi}$). Because no convention existed to identify high-level JAG1 expression in breast cancer tissue analyzed by *in situ* hybridization, we arbitrarily defined $JAG1^{Hi}$ as those tumors expressing JAG1 in the top quartile of the expression range (the same was done for NOTCH1 and NOTCH3, see below). As expected, patients with $JAG1^{Hi}$ tumors had reduced overall survival compared with $JAG1^{Lo}$ tumors (expressing JAG1 at levels within the bottom three quartiles of the expression range; 5-year survival rates of 42% versus 65%), with a median survival time of 50 months as compared with 83 months ($P = 0.01$; Table 4B; Fig. 5*A*). Furthermore, high JAG1 expression was found to be an independent predictor of poor outcome in bivariate analyses with other known predictors of outcome including metastases, patient age, tumor size, node status, ER positivity, and tumor grade (Supplementary Table S3). As menopausal status was not available for these patients, age divided at 50 years was used as a surrogate. Controlling for this variable in the analysis did not alter the significance of JAG1 (data not shown). Similarly, patients with $NOTCH1^{Hi}$ tumors had reduced overall survival compared with $NOTCH1^{Lo}$ tumors (5-year survival rates of 49% versus 64%), with a median survival time of 53 months as

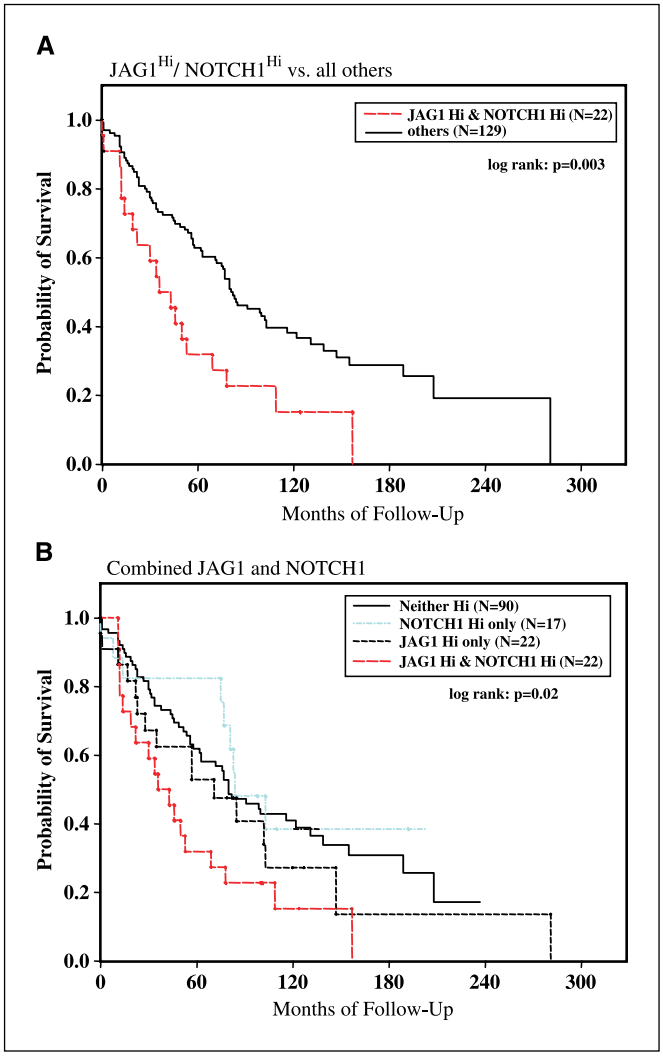

Figure 6. Kaplan-Meier curves showing relationship between high-level JAG1 + NOTCH1 coexpression and overall survival in patients with breast cancer. Patients with tumors coexpressing high levels of JAG1 and NOTCH1 show reduced overall survival in comparison with all other tumors (*A*; $P = 0.003$), or in comparison to $JAG1^{Hi}/NOTCH1^{Lo}$, $JAG1^{Lo}/NOTCH1^{Hi}$, and $JAG1^{Lo}/NOTCH1^{Lo}$ tumors (*B*; $P = 0.02$).

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compared with 91 months (P = 0.02; Table 4B; Fig. 5*B*). A similar trend was observed for NOTCH3, although it did not reach statistical significance (P = 0.08; Table 4B; Fig. 5*C*). The levels of significance of NOTCH1 and NOTCH3 were not altered when age (<50, ≥50) was controlled for in the models.

Elevated coexpression of JAG1 and NOTCH1 defines a subclass of breast cancer with very poor outcome. The levels of expression of JAG1 and NOTCH1 and/or NOTCH3 receptors were not independent of each other. More tumors than expected by random chance coexpressed high levels of JAG1 and either receptor (P = 0.001; Supplementary Table S4). We therefore tested for any relationship between high-level coexpression of JAG1 and NOTCH receptors and patient survival. Indeed, patients harboring tumors with high-level JAG1 and high-level NOTCH1 ($J1^{Hi}$ $N1^{Hi}$) showed worse overall survival than the patients with $JAG1^{Hi}$ or $NOTCH1^{Hi}$ tumors described above, or indeed than all other patients (32% 5-year survival and 40 months median survival for $J1^{Hi}$ $N1^{Hi}$ versus all other patients with 63% 5-year survival and 81 months median survival; P = 0.003; Table 4B; Fig. 6*A*). Furthermore, subgroup analysis suggested that in comparison to patients with tumors expressing low levels of both JAG1 and NOTCH1 ($J1^{Lo}$ $N1^{Lo}$) or high levels of either JAG1 ($J1^{Hi}$ $N1^{Lo}$) or NOTCH1 ($J1^{Lo}$ $N1^{Hi}$), tumors coexpressing high levels of JAG1 and NOTCH1 showed worse overall survival (Table 4B; Fig. 6*B*). Interestingly, these data also suggest the existence of two types of $NOTCH1^{Hi}$ tumors, those that coexpress high levels of JAG1 and those with low levels of JAG1. Patients with these distinct tumor types have dramatically different overall survival.

Finally, analysis of cytokeratin 5/17 expression in the University Health Network tumor set suggested that $JAG1^{Hi}$ tumors were of the basal subtype. Therefore, we tested for cytokeratin 5/17 protein expression in tumors on the tissue microarray. As previously reported, CK5/17-positive tumors were almost exclusively ER-negative (96%). Of the basal subtype tumors, 46% were $JAG1^{Hi}$, a number much higher than expected by chance (test for independence of factors; P = 0.02; see Supplementary Table S2). In addition, we note that 70% of the $JAG1^{Hi}$ tumors were CK5/17-negative, but unlike their CK5/17-positive counterparts, most were ER-positive (75%; see Supplementary Table S2). These data indicate that $JAG1^{Hi}$ tumors belong to at least two of the recently defined breast cancer subtypes.

Discussion

Our data provides the first direct evidence for a relationship between high-level JAG1/NOTCH1 expression and poor overall patient survival in human breast cancer. Our studies predict that a JAG1-NOTCH1 activation loop is functioning to promote tumor formation and progression in the same way that mouse mammary tumor virus–activated Notch1 does in mice (26, 27). Consistent with this, Pece et al. have identified a group of Numb-negative human breast tumors where Notch signaling seems to be activated, at least when cells are cultured *ex vivo* (31). The potential for JAG1-mediated autocrine or juxtacrine Notch signaling in cancer has been established in a number of systems (32–36). In addition, a likely role for JAG1 in aggressive breast tumors fits with its recent implication in prostate cancer metastasis (36).

Molecular profiling experiments have led to the identification of breast cancer subtypes (7, 28, 37, 38). Many of the very aggressive tumors express myoepithelial cell markers, including cytokeratins 5 and 17 (28). Interestingly, some of the $JAG1^{Hi}$ tumors belong to this "basal" subtype (12 of 40), whereas most do not (28 of 40; Supplementary Table S2). We also found that high-level JAG1 expression was an independent predictor of poor survival in bivariate analyses. Thus, high-level JAG1 is likely an important determinant of outcome for breast cancer across subtypes. Indeed, it will be important to determine whether $JAG1^{Hi}$ basal tumors are more aggressive than $JAG1^{Lo}$ basal tumors. Perhaps the basal breast cancer subgroup shows poor overall survival as a result of JAG1-induced Notch activation in some of these tumors. Similarly, high-level expression of JAG1 may help distinguish tumors with reduced survival within other breast cancer subtypes, including erbB2-positive or ER-positive tumors.

Compelling evidence is emerging that abnormal stem cell self-renewal may be an underlying defect in many cancers, including breast cancer (14, 39–41). There is a large body of literature on the role of Notch in stem cell maintenance. For example, Dontu et al. have recently shown that Notch receptor activation regulates self-renewal of mammary stem/progenitor cells in mammosphere cultures (10). Perhaps JAG1, expressed either in differentiated basal tumor cells, in other differentiated tumor cells, or in the tumor stem/progenitor cells themselves can activate NOTCH signaling to promote self-renewal of the tumor-initiating population. In the latter case, a differentiated tumor cell may help to create an artificial "tumor stem/progenitor cell niche." Further studies will be required to test these models. Finally, our data defines a group of human breast tumors that can be identified by screening for Notch ligand and receptor coexpression that may benefit from γ-secretase inhibitor–based therapy (which would block Notch signaling). Such therapies are currently under development for treatment of Alzheimer disease (42), making it easier to test for the efficacy of these inhibitors for treatment of a subset of very aggressive breast tumors as identified in this study.

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