Cancer Research

Plasminogen Activator *uPA* Is a Direct Transcriptional Target of the JAG1-Notch Receptor Signaling Pathway in Breast Cancer

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Abstract

Aberrant activation of the Notch receptor signaling pathway and overexpression of the Notch ligand JAG1 are associated with poor outcome in breast cancer. The plasminogen activator system, which includes urokinase-type plasminogen activator (uPA), has been validated as a marker of recurrence, high metastasis risk and death in breast malignancy. By using microarray profiling of breast cancer cell lines that had undergone siRNA-mediated abrogation of Notch signaling we uncovered a link between activated Notch signaling and uPA expression. An association between elevated expression of the Notch ligand JAG1, uPA, and the basal-like breast cancer subtype was confirmed in breast cancer cell lines. The association between JAG1 and uPA expression persisted in a survey of primary carcinomas of the breast. We found that Notch knockdown reduced transcription of uPA and phenocopied uPA knockdown in breast cancer cells. Through mutational analysis we identified a CBF-1 binding site in the *uPA* promoter that is required for direct transcriptional regulation by Notch. These data suggest that JAG1-induced Notch activation results in breast cancer progression through upregulation of the plasminogen activator system, directly linking these 2 important pathways of poor prognosis.

Cancer Res; 71(1); 277–86. ©2011 AACR.

Introduction

Despite the success of targeted therapies such as antiestrogens and trastuzumab, breast cancer ranks second among causes of cancer death in North American women (1). The identification of molecular pathways that are activated in breast cancer and the development of novel strategies that target them, will improve clinical outcome.

The plasminogen activator system is involved in multiple physiological and pathologic processes including cell migration, angiogenesis, wound healing, embryogenesis, tumor growth, and metastasis. The urokinase-type plasminogen activator (uPA) binds to its receptor (uPAR), which facilitates conversion of plasminogen to plasmin. Plasmin, either directly or indirectly through metalloproteinases (MMP), can degrade components of the extracellular matrix contributing to cancer cell invasion and metastases. In breast cancer, high levels of uPA and/or its inhibitor (PAI-1) significantly correlate with tumor aggressiveness and poor outcome, and have achieved level-1 evidence as clinically useful prognostic indicators [reviewed by Annecke et al. (2)]. Breast cancer patients with high levels of uPA and/or PAI-1 have an enhanced benefit from adjuvant chemotherapy compared with patients with tumors that express low levels, showing the predictive value of the urokinase system (3).

The Notch signaling cascade is highly conserved and plays a crucial role in stem cell self-renewal, cell fate determination, epithelial cell polarity, adhesion, cell division, and apoptosis (4). Mammals have 4 Notch receptors (NOTCH1–4) that interact with 5 Notch ligands (DLL1, 3, 4; JAG1, 2). Notch ligand–receptor interaction on neighboring cells leads to a series of proteolytic events including a presenilin-protease (γ-secretase)–mediated cleavage that liberates the active cytoplasmic domain fragment, intracellular NOTCH (N^{IC}), from the plasma membrane. N^{IC} translocates to the nucleus, in which it engages the DNA binding protein CBF-1/RBPJκ resulting in replacement of a multiprotein corepressor complex with a coactivator complex and transcription of target genes (5).

Several lines of evidence suggest that Notch activation contributes to the pathogenesis of human breast cancer. Activated Notch signaling has been observed in breast cancer cell lines and primary breast cancers (6–8). In primary breast cancer, increased expression of NOTCH1 has been observed in

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

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doi: 10.1158/0008-5472.CAN-10-2523

tumors that overexpressed H-Ras (9) and the expression of Numb, a negative regulator of the Notch pathway, was found to be inversely related to Notch activation, tumor grade, and the growth inhibitory effects of Notch antagonists (10). High-level expression of JAG1 and/or NOTCH1 mRNA correlates with high tumor grade and poor outcome and is an independent prognostic indicator (11–13). Consistent with these findings, elevated expression of N^{IC} in ductal carcinoma *in situ* lesions predicts early recurrence (14).

Despite the evidence that Notch plays a prominent role in breast cancer, few direct Notch transcriptional targets have been identified in this context. In this study, we show that JAG1-induced Notch activation directly regulates the expression of uPA.

Materials and Methods

JAG1–uPA expression correlation in multiple breast cancer gene expression data sets

Publicly available gene expression data were obtained from 3 series of breast cancer cell lines, Hoeflich et al. (15), Hollestelle et al. (16), and Neve et al. (17), and analyzed as described in Supplementary Methods. In each data set, 870 unique variable genes underwent hierarchical clustering with Pearson correlation metric using Cluster 3.0 (18) and visualized using Java Treeview (19). Spearman rank correlation was calculated using StatPlus v2009 build 5.8.1.0 (AnalystSoft Inc.).

Tissue microarrays and immunohistochemistry

Analysis of JAG1 by immunohistochemistry (IHC) was performed as previously described (11), and for uPA as described in Supplementary Methods. Protein expressions of JAG1 and uPA were quantified by application of the Allred score as previously described (11). χ^2 Test was used to look for a relationship between the levels of JAG1 and uPA expression. $P \leq 0.05$ was considered statistically significant.

Cell culture, Western blotting, antibodies, γ-secretase inhibitors, and siRNA

Cell lines were purchased from American Type Culture Collection. Anti-NOTCH1 (sc-6014-R), anti-JAG1 (sc-8303), anti-NOTCH3 (sc-5593), anti–NF-κβ p50 (sc-7178), anti–NF-κβ p65 (sc-372), and anti-actin horseradish peroxidase (HRP; sc-1615 HRP) were purchased from Santa Cruz Biotechnology, Inc. and anti-uPA from Chemicon. γ-Secretase inhibitor (GSI) IX/[N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester] and GSI I were purchased from Calbiochem and used at 50 μmol/L and 1.5 μmol/L, respectively. For siRNA sequences, see Supplementary Table S4. Unless otherwise specified, siRNA was used at 50 nmol/L.

Gelatin zymography

Cells were transfected with siRNA, and after 24 hours the media was removed and replaced with serum-free media for 48 hours. Conditioned media was collected and concentrated with Amicon Ultra Centrifugal filters (Millipore UFC801024). Gelatin zymography was performed as described by Heussen and Dowdle (20).

Cell invasion and migration assays

Invasion and migration assays were performed in transwells coated with or without Matrigel (BD Biosciences), respectively, according to the manufacturer's specifications (see Supplementary Methods).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT Express kit (Active Motif) according to the manufacturer's specifications. Details are provided in Supplementary Methods and Supplementary Table S4.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed by incubating 5 μg of nuclear protein extract from transfected cells with 50 fmoles of IRDye 700–labeled oligonucleotide probe. For NF-κB, a predesigned probe was purchased from LI-COR (#829–07924). Where indicated, 10 pmoles of cold competitor oligonucleotide probe was added to the binding reaction (range of 0–10 pmole in the dose–response assay).

Luciferase reporter assay

The *uPA* promoter (RDB#5547; KM2L-phUPA) was provided by RIKEN BRC, which is participating in the National Bio-Resources Project of the MEXT, Japan. A *Sac*I/*Sma*I fragment (nucleotides −2495 to −393) was inserted into the pGL3 luciferase expression vector (Promega) to generate pGL3UPA. Nucleotides −1161 and −1162 (CA) within CBF-1 binding site (CBS) B were mutated to TC (pGL3UPAmutB). The 293 cell line was cotransfected with 200 ng of pGL3UPA, 100 ng of cytohN1pcDNA3 (kindly supplied by Dr. S. Artavanis-Tsakonas) or pBabeN3ICD-V5 [kindly supplied by Dr. T. L. Wang (21)], and 20 ng of pcDNATM1.2/V5-GW/lacZ in a total of 1 mL medium. After 24 hours, luciferase activity was measured (Luciferase Assay System; Promega Corporation) and normalized to β-galactosidase activity (Invitrogen). For HCC1143, cells were transfected with 20 pmoles of siRNA in 0.5 mL media. Two days following transfection, 400 ng of either pGL3UPA or pGL3UPAmutB and 40 ng of pcDNATM1.2/V5-GW/lacZ were cotransfected, and luciferase and β-galactosidase activity measured at 24 hours.

Results

Notch-associated gene expression patterns in breast cancer cell lines

A tumor-promoting role has been proposed for activated Notch signaling in several malignancies including hematologic malignancies and tumors of the gut, prostate, breast, and skin (22). We have previously shown an association between JAG1, NOTCH1, and NOTCH3 expressions and poor outcome in breast cancer (12). Emerging evidence suggests that activated Notch signaling is associated with estrogen receptor (ER)-negative breast cancers including those of the basal-like subtype (13, 23). To determine tumorigenic regulatory programs that are activated by Notch in ER-negative breast tumors, we used expression arrays to identify differentially expressed Notch target genes in MDA MB231 and HCC1143

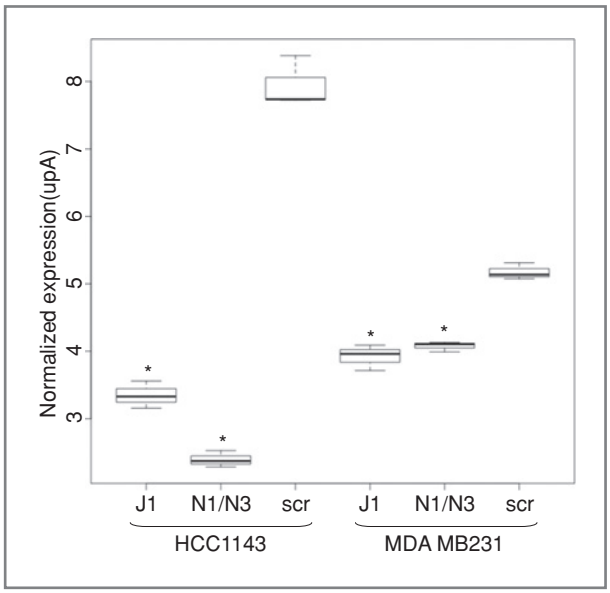

Figure 1. Boxplots of normalized uPA expression levels assayed on Agilent 44k Whole Human Genome chips after treatment of MDA MB231 or HCC1143 cell lines with JAG1 (J1) or NOTCH1 (N1) and NOTCH3 (N3) siRNA. Three replicate measurements were performed per group (Supplementary Methods). *, $P < 0.05$ compared with the scrambled (scr) control.

cells after siRNA-mediated knockdown of JAG1 or NOTCH1 and NOTCH3. We have previously ascertained that JAG1, NOTCH1, and NOTCH3 siRNA treatments effectively reduce protein expression of their targets, N^{IC} levels and proliferation in these cell lines (24). The normalized and filtered expression array data were subjected to a 2-way ANOVA (with cell line and siRNA treatment being the 2 factors). In total, 680 genes were found to be significantly differentially regulated with a greater than 2-fold change in 1 or both knockdown conditions (JAG1 or NOTCH1/NOTCH3) or cell lines (Supplementary Fig. S1; Supplementary Table S1; Supplementary Methods). Within this set of 680 genes, we identified previously described Notch target genes including *CCND1* (24). Among the genes identified were members of the urokinase system including *uPA* (Fig. 1) and *MMP9*.

Correlation of JAG1 and uPA in breast cancer cell lines and primary breast cancer

On the basis of the expression array analysis we sought to identify a correlation between uPA and Notch in additional breast cancer cell lines. Gene expression data from 51 breast cancer cell lines (15) were normalized, median centered, and analyzed by unsupervised hierarchical clustering based on a set of top variable genes (Materials and Methods). The results showed a significant correlation between *JAG1* and *uPA* gene expression with the highest levels of JAG1/uPA mRNA coexpression observed in ER-negative cell lines, including those of basal-like, CD24 low/mesenchymal, and $HER2^{+}$/basal subtypes (Fig. 2A). A significant correlation between JAG1 and uPA expression was confirmed in 2 additional breast cancer cell line expression array data sets (Table 1).

To determine if JAG1 and uPA expression correlate in primary carcinoma of the breast, uPA IHC was performed on a breast cancer tissue microarray for which we had previously reported JAG1 protein expression (11). These analyses revealed a significant association between JAG1 and uPA expression levels in primary breast cancer (Fig. 2B; Supplementary Table S2). Next, JAG1 and uPA were evaluated in 4 publicly available breast tumor microarray data sets and a significant correlation in patterns of expression was found in 1 set (Supplementary Table S3). We hypothesized that analysis of microarray data obtained from whole tumor extracts could be confounded by the presence of transcripts from nonmalignant stromal tissue, masking a potential association between JAG1 and uPA expression in the discordant data sets. To determine if discordant JAG1/uPA expression in whole tumor extracts may be in part due to stromal expression of uPA, we utilized a previously described stromal metagene (25) to select a subset of tumor samples with the lowest stromal gene expression signature (26). Indeed, this analysis showed a correlation between JAG1 and uPA expression in "stromal-low" samples that were of high histologic grade and ER- and HER2-negative (Fig. 2C–E).

uPA expression level and enzymatic activity are dependent on Notch ligand and receptor expression

To validate the microarray findings, we tested the effect of NOTCH3, NOTCH1, or JAG1 siRNA or GSI on uPA expression and enzymatic activity in breast cancer cells (Fig. 3). The ER-negative/basal-like breast cancer cell lines HCC1143, MDA MB231, and MDA MB468 were used for this purpose. To reduce the possibility that observations were due to off-target effects, several distinct siRNA preparations were tested for each gene. NOTCH3 siRNA achieved efficient target knockdown and a corresponding reduction in uPA expression (Fig. 3A). Despite levels of NOTCH3 expression detectable only by PCR (24), MDA MB231 cells were dependent on this receptor for efficient uPA expression (Fig. 3A, lanes 6–9; Supplementary Fig. S2). In all 3 cell lines tested, uPA enzymatic activity was reduced in accordance with reduction in uPA expression. JAG1 and NOTCH1 silencing similarly reduced uPA expression and activity in all 3 cell lines, although the effect on uPA activity was not as pronounced in MDA MB468 cells (Fig. 3B and C). Consistent with the hypothesis that Notch regulates uPA expression, treatment of breast cancer cells with GSI significantly reduced uPA expression and enzymatic activity (Fig. 3D).

Inhibition of Notch signaling disrupts cell migration and invasion

Our observation that uPA activity was regulated by Notch, prompted us to compare the effect of Notch and uPA knockdown on breast cancer cell proliferation, migration, and invasion. Standard Matrigel invasion and migration assays after siRNA-mediated NOTCH1, NOTCH3, JAG1, or uPA silencing were performed in MDA MB231 cells (Fig. 4). We found that knockdown of the Notch signaling pathway phenocopied knockdown of uPA. At 52 hours posttransfection, knockdown of NOTCH3, JAG1, and uPA reduced migration and invasion of

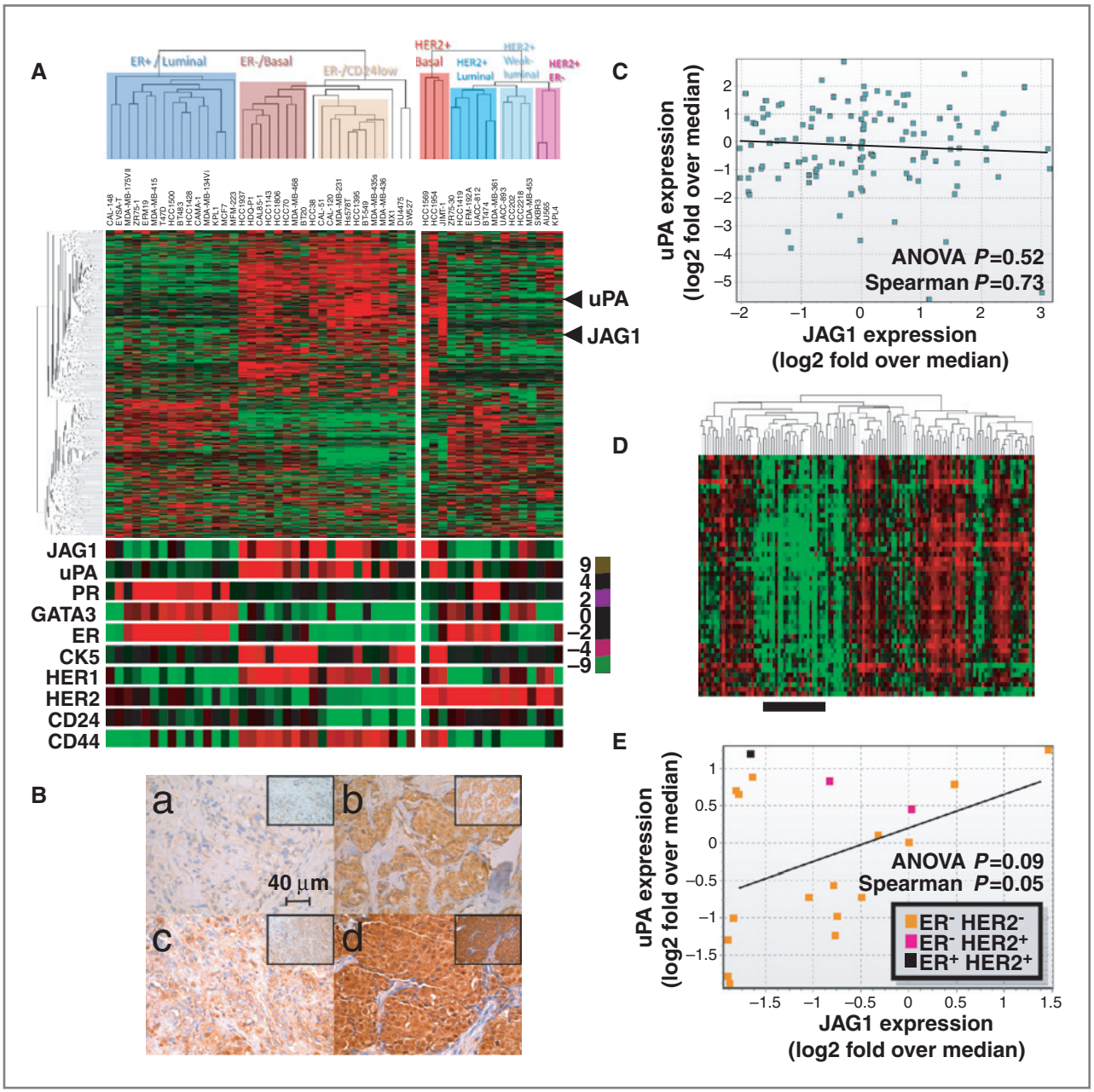

Figure 2. Coexpression of JAG1 and uPA in breast cancer cell lines and primary breast cancer. A, to visualize the pattern of *JAG1*/*uPA* gene expression in the context of molecular subtypes of breast cancer cell lines, unsupervised hierarchical clustering of median-centered gene expression values was performed using a set of 870 genes of highest variability between 51 breast cancer cell lines (see Materials and Methods). Shown is a heatmap of gene expression patterns from the entire set of 870 genes and magnified data from *JAG1*/*uPA* and selected genes characteristic of molecular subtypes. Fold-change relative to the median value is indicated by the colored bar. B, photomicrographs of tissue microarray cores following IHC for JAG1 (a and b) or uPA (c and d). Note: compared with Patient 1 (a and c), tumor from Patient 2 (b and d) shows high level coexpression of JAG1 and uPA. Photomicrographs are shown using ×40 objective and ×20 objective (inset). C, scatter plot diagram of *uPA* and *JAG1* gene expression compared in a microarray data set comprised of 127 primary breast cancers (26) shows a poor correlation between uPA and JAG1 (linear regression ANOVA and Spearman rank correlation *P* values shown). D, unsupervised hierarchical clustering of the 127 tumors using an established stromal metagene (50 genes; ref. 25) identifies a subset of 19 tumors (black bar) with a "low" stromal signature. E, relationship of *uPA* and *JAG1* gene expression in 19 "stromal-low" tumor samples. Note: 18 of 19 samples are negative for ER expression.

MDA MB231 cells without affecting proliferation. Knockdown of NOTCH1 reduced invasion without significantly affecting migration.

CBF-1 binds to the *uPA* promoter/enhancer

To determine the mechanism by which Notch signaling regulates uPA expression, we interrogated a 2,500 bp region

Table 1. Correlation between JAG1 and uPA expression in breast cancer cell lines

Sample set	Samples	Spearman *R*	*P*
Genentech	51	0.47	0.0005
Netherlands	41	0.38	0.02
UCSF	54	0.33	0.01

NOTE: Spearman rank correlation *R* values and significance *P* level for JAG1 and uPA mRNA expression were obtained from 3 independent breast cancer cell line gene expression data sets: Hoeflich et al. (ref. 15; Genentech), Hollestelle et al. (ref. 16; Netherlands), and Neve et al. (ref. 17; UCSF).
Abbreviation: UCSF, University of California, San Francisco.

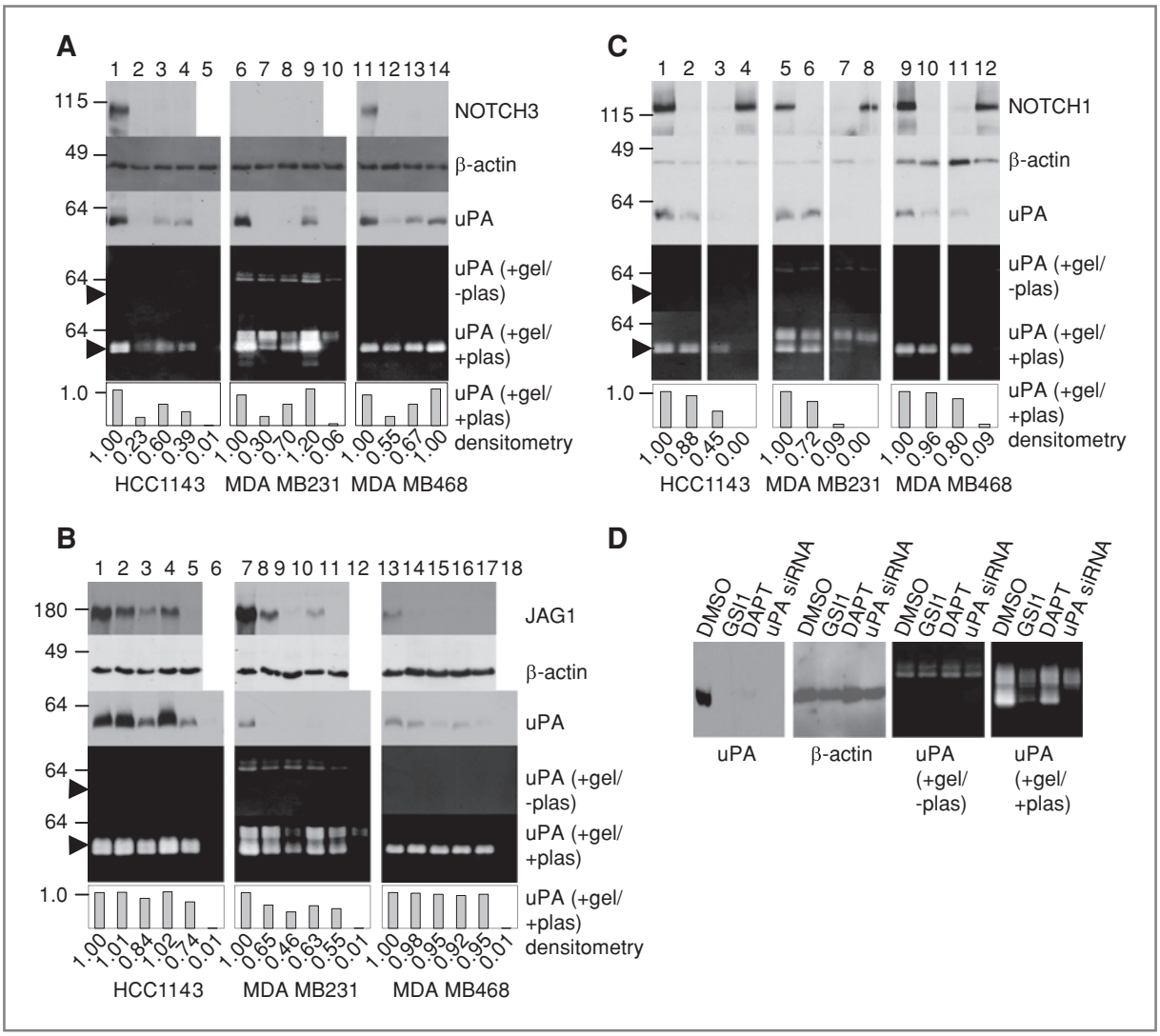

Figure 3. Effect of NOTCH3, JAG1, and NOTCH1 siRNA treatment or GSI on uPA expression and activity in breast cancer cell lines. Expression of uPA in serum-free conditioned media from HCC1143, MDA MB231, and MDA MB468 cells treated with either control scrambled (scr), uPA, NOTCH3 (A), JAG1 (B), NOTCH1 (C), siRNA or MDA MB231 cells treated with GSI (GSI1 or DAPT; D). The enzymatic activity of uPA was determined by quantifying gelatin digestion in the presence of plasminogen [uPA (+gel/+plas), see Materials and Methods] as measured by densitometry. Conditions in which plasminogen was eliminated [uPA (+gel/−plas)] were included as a negative control. Expression of β-actin is included as a loading control. Mw markers are shown in kilodaltons. A, scr (lanes 1, 6, 11); N3 siRNA-A (lanes 2, 7, 12); N3 siRNA-C (lanes 3, 8, 13); N3 siRNA-12 (lanes 4, 9, 14); uPA siRNA (5, 10). B, scr (lanes 1, 7, 13); J1 siRNA-A (lanes 2, 8, 14); J1 siRNA-B (lanes 3, 9, 15); J1 siRNA-54 (lanes 4, 10, 16); J1 siRNA-55 (lanes 5, 11, 17); uPA siRNA (6, 12, 18). C, scr (lanes 1, 5, 9); N1 siRNA H08 (lanes 2, 6, 10); N1 siRNA-A (lanes 3, 7, 11); uPA siRNA (lanes 4, 8, 12). DMSO, dimethyl sulfoxide.

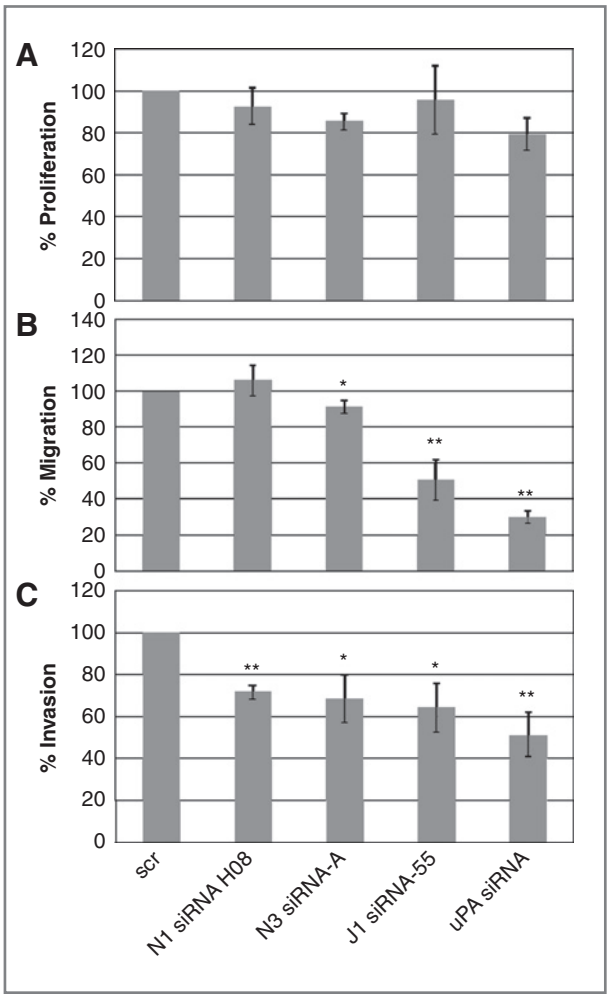

Figure 4. Inhibition of Notch signaling disrupts MDA MB231 cell migration and invasion. Comparison of proliferation (A), migration (B), and invasion (C) of cells transfected with scrambled (scr), NOTCH1 (N1 siRNA H08), NOTCH3 (N3 siRNA-A), JAG1 (J1 siRNA-55), and uPA (uPA siRNA) siRNAs. Percent of cells relative to the control is shown; bars represent SE (*, $P < 0.05$; **, $P < 0.01$ compared with the control).

upstream of the start codon of the human *uPA* promoter/enhancer for putative CBS. Five putative CBS were identified: 4 low-affinity sites (RTGRGAR; sites A, D, E, and F, Fig. 5A) and 1 high-affinity site (YGTGRGAA; site B; ref. 27). Of these, sites B and F showed significant sequence conservation between species (Fig. 5B).

To explore whether the *uPA* gene is a direct Notch transcriptional target, we used complementary approaches. First, we undertook ChIP assays of lysates from HCC1143 cells using antibodies against $N1^{IC}$ or $N3^{IC}$. The immunoprecipitated chromatin fragments were analyzed by PCR using 2 primer sets; set 1 targeted site B and set 2, site F (Fig. 5A). Enrichment for NOTCH1 or NOTCH3 protein–chromatin complexes was detected by primer set 1 only, indicating direct binding of these receptors to the *uPA* promoter/enhancer in a region containing the high-affinity site B (Fig. 5C).

The ability of CBF-1 to bind directly to site B was examined by EMSA. Although a probe derived from putative CBS B (probe B) was able to form a DNA–protein complex, probe C, which contains an incomplete CBS consensus, was unable to do so (Fig. 5D). Formation of the probe B–protein complex was competitively inhibited with excess molar amounts of unlabeled wild-type but not mutant probe B. The identity of CBF-1 in the probe B–protein complex was confirmed by showing the inability of the complex to form in nuclear extracts derived from cells that had undergone siRNA-mediated CBF-1 knockdown (Fig. 5E).

To investigate whether Notch could directly modulate uPA expression, we first analyzed induction of the *uPA* promoter/enhancer by $N1^{IC}$ or $N3^{IC}$ in luciferase reporter assays in 293 cells (Fig. 6A). Both $N1^{IC}$ and $N3^{IC}$ resulted in an increase in *uPA* promoter activity. Conversely, knockdown of NOTCH1, NOTCH3, or JAG1 in HCC1143 cells resulted in a decrease in *uPA* promoter activity ($P < 0.005$; Fig. 6B). A promoter/enhancer containing a mutant site B, showed a significant reduction in luciferase activity compared with wild-type ($P < 0.005$) and this reporter was refractory to NOTCH3 or JAG1 siRNA knockdown. Notably, only NOTCH1 siRNA treatment further reduced reporter activity in the mutant ($P < 0.05$). These data identify site B as a Notch-responsive CBS, and suggest that there may be additional NOTCH1-responsive CBS in the *uPA* promoter/enhancer.

Notch can regulate NF-κB activity (28–30) and the *uPA* promoter/enhancer contains binding sites for NF-κB (31). Therefore, we explored the possibility that uPA activity was also indirectly controlled by Notch through NF-κB. We found that siRNA-mediated knockdown of JAG1, NOTCH1, or NOTCH3 in MDA MB231 cells did not affect the total p50 and p65 NF-κB protein levels (Supplementary Fig. S3A) or NF-κB DNA-binding activity as measured by EMSA (Supplementary Fig. S3B).

Discussion

Evidence supporting a role for abnormal Notch signaling in the progression of human breast cancer continues to accumulate. Having previously shown that elevated expression of JAG1 and NOTCH1 correlate with poor outcome in breast cancer (12), we performed microarray profiling to identify effectors of Notch that drive the aggressive nature of Notch-activated breast cancer. Direct Notch target genes in breast cancer discovered to date include the cell cycle regulator, *CCND1* (24), *Myc* (32), the Inhibitor of Apoptosis gene family member, *Survivin* (23), and *Slug* (33), a regulator of epithelial–mesenchymal transition. There is abundant evidence that the plasminogen activator system plays an important role in breast cancer growth, invasion and, metastases (2), and here we provide evidence that places Notch directly upstream of this system. We show that uPA transcription is regulated through canonical Notch signaling, and that JAG1 and uPA mRNA expression correlate in multiple breast cancer gene expression data sets. These data underscore the relevance of Notch-mediated uPA expression to the development of human breast cancer.

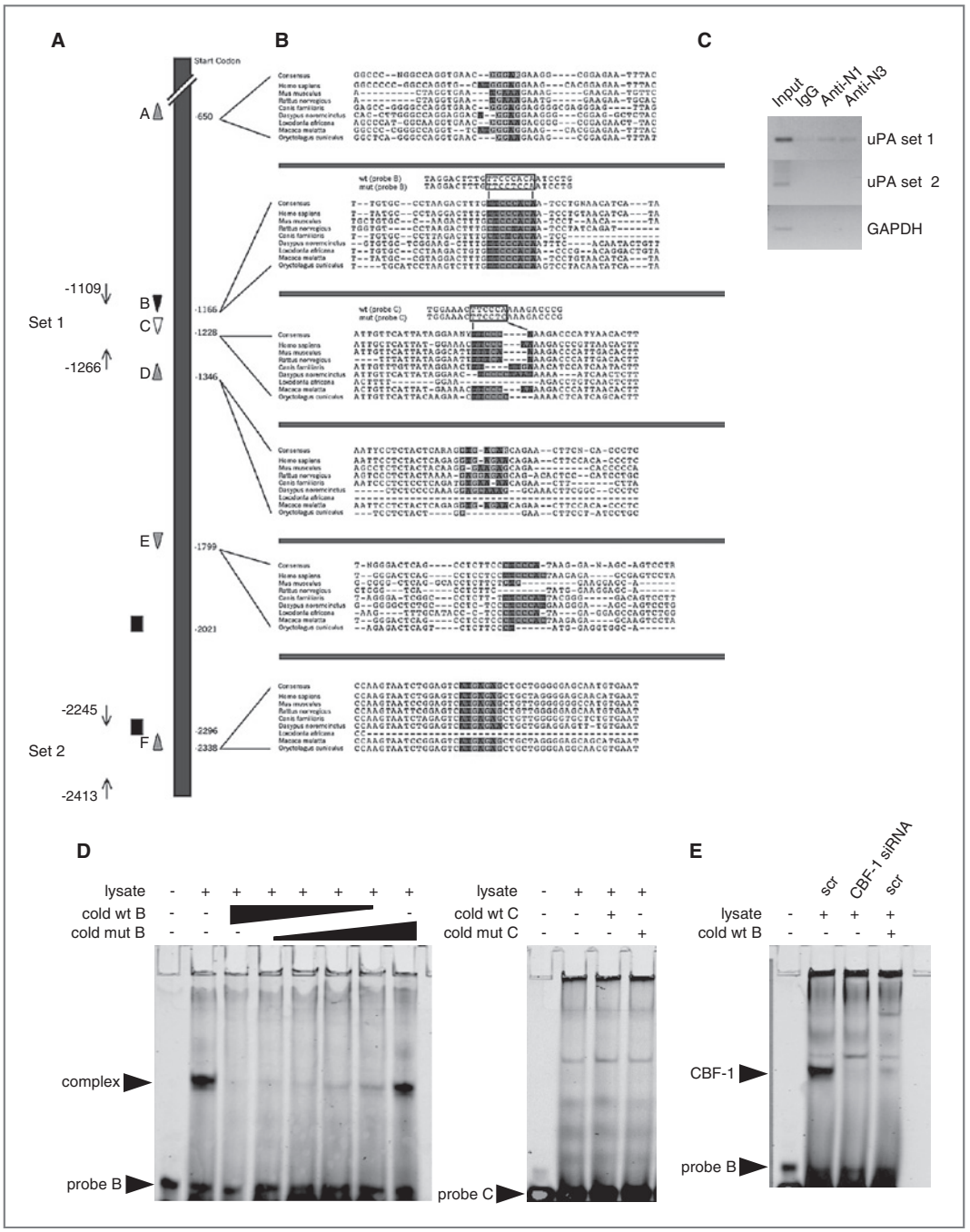

Figure 5. Identification of a CBS in the human *uPA* promoter/enhancer. A, schematic representation of the human *uPA* promoter/enhancer indicating the location of putative CBS. On the basis of a search for the core binding motif TGRGAR, a single high-affinity CBS (YGTGRGAA; black triangle, site B) and multiple low-affinity CBS (RTGRGAR; grey triangles, sites A, D, E, and F) are identified. Site C (open triangle) is a partial CBS, targeted as a negative control in EMSA (Fig. 5D). The locations of previously described NF-κB binding sites (rectangles) are also indicated (31). The 5′ locations of transcription factor binding sites are indicated relative to the start of translation of uPA. The location of the 5′ ends of the primer pairs (sets 1 and 2) used in the ChIP analyses are indicated. B, interspecies alignment of CBS. Putative CBS are highlighted. The sequences of the sense strands of wild-type (wt) and mutant (mut) oligonucleotide probes that target sites B (probe B) and C (probe C) used in EMSA are shown. C, ChIP assay of NOTCH binding to the human *uPA* promoter/enhancer. ChIP with NOTCH1 (Anti-N1), NOTCH3 (Anti-N3), and control, nonspecific (IgG) antisera from HCC1143 cell lysate (Input). QRT-PCR was performed using primer sets 1 and 2 of the *uPA* promoter (Fig. 5A). Assays with primers that target an unrelated promoter (*GAPDH*) are included as a negative control. D, the sequence identified in the human *uPA* promoter/enhancer (site B, Fig. 5A) forms a complex with components from MDA MB231 nuclear extract. EMSA analysis was performed by incubating IRDye 700–labeled oligonucleotide probe (probe B or C) with nuclear extract from MDA MB231 cells. Complex formation was analyzed in the presence of competing wt or mut unlabeled oligonucleotide probe (0–10 pmoles). E, EMSA analysis in the presence of nuclear extracts from cells treated with either scrambled (scr) or CBF-1 siRNA. CBF-1, the CBF-1–probe B complex.

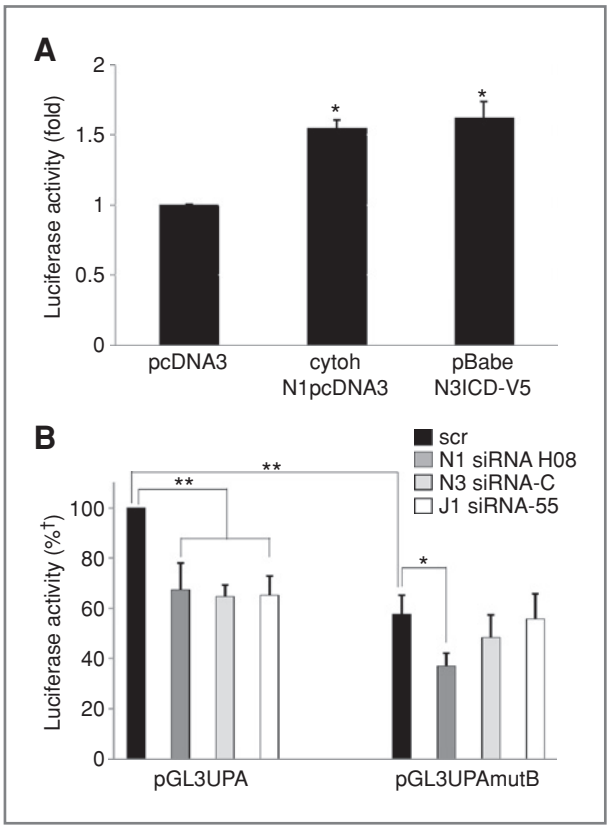

Figure 6. CBS B is a Notch-responsive element and is required for normal *uPA* promoter/enhancer activity. A, luciferase activity in 293 cells cotransfected with pGL3UPA and either pcDNA3, or plasmids expressing either an $N1^{IC}$ (cytohN1pcDNA3) or $N3^{IC}$ (pBabeN3ICD-V5; described in Materials and Methods). Results are expressed as the fold-induction above pcDNA3 (control)-treated cells, normalized to β-galactosidase activity and as SEM of 4 determinations (*, $P < 0.0001$). B, luciferase activity in HCC1143 cells cotransfected with either pGL3UPA or pGL3UPAmutB and either scrambled (scr) siRNA or siRNA targeting JAG1, NOTCH1, or NOTCH3. †, results are expressed as % of luciferase activity in pGL3UPA/scr-transfected cells, normalized to β-galactosidase activity and as the SEM of 4 determinations (*, $P < 0.05$; **, $P < 0.005$).

Our analysis of breast cancer cell lines shows a correlation between JAG1 and uPA expression, with the coexpression of the highest levels of JAG1 and uPA occurring in ER-negative/basal subtype cell lines. In primary breast cancer, the analysis of JAG1/uPA coexpression is complicated by the fact that uPA is expressed in multiple compartments including tumor epithelium, stroma, and endothelium (34). Focusing on a "stromal-low" subset allowed us to show JAG1/uPA coexpression in primary breast cancer. Consistent with this finding, our immunohistochemical analysis of a breast cancer tissue microarray (TMA) which allowed assessment of the tumor epithelial compartment, showed significant JAG1/uPA coexpression. Our cell line analysis suggests that elevated JAG1/uPA coexpression is a defining feature of basal-like breast cancer and its verification in primary breast cancer will require future immunohistochemical analysis of a large primary breast cancer TMA.

Although recent reports have showed that NOTCH1 stimulates expression of uPA in carcinomas of the prostate and cervix (35, 36), our study is the first to identify direct regulation of uPA expression by Notch through at least 1 functional CBF-1 binding consensus (site B) within the uPA promoter/enhancer. However, the finding that NOTCH1 siRNA knockdown could further reduce expression from a reporter containing a mutation within CBS B, suggests the existence of additional NOTCH1-responsive CBS within the *uPA* promoter/enhancer. Further studies are underway to fully characterize the Notch-responsive elements within the *uPA* promoter/enhancer.

Regulation of uPA expression has been previously shown to occur through multiple transcription factors including NF-κB (31), activator protein-1 (37), and FOXO3a (38). Our analyses in breast cancer cell lines show significant regulation of uPA expression by Notch; indeed, uPA expression could be reduced to low or undetectable levels in breast cancer cell lines treated with inhibitors of the γ-secretase complex or after siRNA-mediated Notch ligand/receptor knockdown emphasizing the importance of Notch activation to uPA expression. Consistent with these biochemical findings, uPA knockdown achieved either directly with uPA siRNA or indirectly through Notch ligand/receptor knockdown, resulted in a phenotype characterized by defects in both migration and invasion, processes in which the urokinase system is known to play a central role (39).

Cancer mortality in most instances results from tumor invasion and hematogenous or lymphatic metastases. The drivers of these key tumor activities are important to identify because they may serve as predictive/prognostic biomarkers or as therapeutic targets. This is particularly urgent in the case of basal-like breast cancer, a subtype associated with poor outcome and for which there are currently no recommended targeted therapies. Several lines of evidences including data obtained from breast cancer cell lines and from patient samples suggest that Notch activation is associated with breast cancer of the basal-like subtype (12, 13, 23, 24). Our findings suggest a mechanism whereby JAG1/Notch-driven uPA expression promotes the aggressive behavior of basal-like breast cancer. Thus, our data provide a rationale for the use of GSI-based therapy in this breast cancer subtype, and identifies the potential of uPA as a predictive biomarker in Notch-activated breast cancer. Therapies that target γ-secretase are currently under evaluation for the treatment of breast cancer (40); however, untoward side effects may limit their usefulness. Indeed, because Notch signaling is involved in gastrointestinal (GI) homeostasis (41), treatment with GSIs can result in severe cytotoxicity within the GI tract (42). Further clinical trials designed to test the efficacy and safety of Notch pathway inhibition may influence breast cancer treatment as we enter an era of personalized cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We thank Mr. Anoosh Rakhshandeh for statistical analyses.

Grant Support

This study was supported by funds to M. Reedijk from the Canadian Breast Cancer Foundation and the Canadian Institutes of Health Research. This research was funded in part by the Ontario Ministry of Health and Long Term Care.

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Received July 14, 2010; revised September 30, 2010; accepted October 7, 2010; published online January 3, 2011.

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Cancer Res 2011;71:277-286.