Dominant-Negative Notch3 Receptor Inhibits Mitogen-Activated Protein Kinase Pathway and the Growth of Human Lung Cancers

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Abstract
Notch3 is a member of an evolutionarily conserved family of cell surface receptors important in cell-fate determination in both vertebrates and invertebrates. Significant data support the role of Notch pathway in cancer development, although the conflicting role of Notch signaling pathways in tumorigenesis suggests that its action is highly context-dependent. Furthermore, although Notch receptors signal primarily through the regulation of hairy enhancer of split (HES) and HES-related (HRT) genes, they are known to crosstalk with other signaling pathways, including the epidermal growth factor (EGF) and the mitogen-activated protein kinase pathways. Whereas much is known about the role of Notch1 in human cancer, the role of Notch3 in epithelial tumors, such as lung carcinomas, has not been well established. In this study, we show that Notch3 is expressed in 80 of 207 (39%) resected human lung tumors and that its expression is positively correlated with EGF receptor expression. Inhibition of the Notch3 pathway using a dominant-negative receptor dramatically reduces growth in soft agar and increases growth factor dependence. We also find that Notch inhibition increases sensitivity to EGF receptor tyrosine kinase inhibition and decrease in phosphorylation of the mitogen-activated protein kinase. These observations support a role for Notch3 signaling in lung cancer, and one potential mechanism of maintaining the neoplastic phenotype is through the modulation of the EGF pathway. (Cancer Res 2005; 65(9): 3555-61)

Introduction
The Notch signaling pathway is an evolutionarily conserved, intracellular signaling mechanism important in cell fate determination during the development of both vertebrates and invertebrates. The basic picture emerging from previous studies in Drosophila melanogaster is that the Notch protein is expressed on the cell surface as a heterodimeric receptor. Upon binding with its ligand, Delta or Serrate, Notch is activated via a series of proteolytic cleavages, resulting in the release of the intracellular domain. In Drosophila, the intracellular domain translocates to the nucleus and binds to Suppressor of Hairless (Su(H)). The binding of Notch intracellular domain and Su(H) results in the transcription of enhancer of split (E(spl)) genes, which in turn down-regulates Achaete-Scute, a gene important in the segregation of neuronal and epidermal differentiation (for an excellent review, see ref. 1).

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cell lines, a direct role of Notch3 in lung cancer tumorigenesis has not been shown. In this study, we show that Notch3 is frequently expressed in a subset of resected human lung cancers. Our studies also show that inhibition of Notch3 signaling using a dominant-negative receptor attenuates the malignant phenotype, increases tumor dependency on exogenous growth factors and can sensitize tumors to EGFR inhibition. Our data will provide compelling evidence for the direct role of Notch3 in the tumorigenesis of epithelial carcinomas such as lung carcinomas.

Materials and Methods

Cell lines and transfections. The Notch3-expressing cell lines, HCC2429 and H460, were maintained in RPMI with 10% FCS as described previously (20). The immortalized lung epithelial cell line, BEAS-2B, was obtained from the American Tissue Culture Collection (Manassas, VA) and maintained in DMEM in 10% FCS. The pDNA3.1 dominant-negative construct was transfected into HCC2429 using LipofectAMINE (Invitrogen, Carlsbad, CA) according to manufacturer’s recommendation. After 48 hours, the cells were selected using G418. Incorporation of the dominant-negative construct was determined using antibody detection of the V5 epitope tag. In some studies, the cell lines were maintained in RPMI supplement with recombinant EGF (R&D System, Minneapolis, MN) at 100 ng/mL. The EGFR tyrosine kinase inhibitor AG1478 was obtained from Calbiochem (San Diego, CA).

Retroviral vector construction and infection. The dominant-activator construct was excised from pDNA3.1-DA and subcloned into the retroviral vector pBabe-puro using compatible BamHI and EcoRI ends. Recombinant viruses were generated by first transfecting the helper-free, ecotropic packaging Phoenix cell lines using LipofectAMINE 2000 (Invitrogen). Viral supernatant derived from these cells was filtered (pore size, 0.45 μm) and used to infect BEAS-2B. After 48 hours of infection, the cells were split and selected using 2 μg/mL puromycin with 10% DMEM.

Plasmids. The extracellular domain (residues 1-1665) of human Notch3 (dominant-negative) and intracellular domain (residues 1663-2321) of Notch3 (dominant-activating) were generated by PCR, sequence-confirmed, and subcloned into a pDNA1.5/V5-His expression plasmid which has bicistronic expression of V5 and histidine epitope tags and inserted genes under cytomegalovirus promoters. Both expression constructs were amplified by PCR from plasmid pSG5-Notch3 (a gift from Dr. A. Joutel, Faculté de Medecine Lariboisière, Paris, France). Because a nucleotide difference at position 5233 resulted in the change of amino acid in the SG5-Notch3 template when compared with Genbank HsU979669, we excised a fragment from the dominant-negative construct and exchanged it with another fragment amplified from HCC2429-derived cDNA.

Antibodies, immunohistochemistry, and Western blot experiments. Antibodies to phospho-p44/42, p44/42, phospho-akt, and Akt were obtained from Cell Signaling (Beverly, MA) and used in accordance with the manufacturer’s instructions. Antibodies to total MEK1/2 and β-actin were obtained from Zymed Laboratories (San Francisco, CA) and Sigma-Aldrich (St. Louis, MO), respectively. The anti-HES-1 antibody was obtained from CéMines (Golden, CO). For Western blot analysis of mitogen-activated protein kinase (MAPK) and MAP/ERK kinase (MEK) activation, the cells were maintained in serum-free medium for 24 to 48 hours prior to EGF or serum stimulation. Inhibitors U0126 and AG1478 were added 2 hours prior to stimulation. Cells were harvested after the designated time points accordingly.

Immunohistochemical detection of EGFR receptor was accomplished using monoclonal, prediluted antibody (clone 3G7) from Zymed Laboratories according to the manufacturer’s recommendation. The Notch3 antibody 1E4 used for immunohistochemistry (a gift from Dr. A. Joutel) has been described previously (22). Immunohistochemistry was done using the Avidin-Biotin Complex Vectastain kit (Vector Laboratories, Burlingame, CA) with the following modifications: the slides were treated with 3% H2O2 to quench endogenous peroxidase activity, blocked with 10% fetal bovine serum, and incubated with primary antibody (1:5 dilution) overnight at 4°C. The sections were counterstained with Gill’s hematoxylin solution. To determine the frequency of Notch3 expression in primary resected lung tumors, we used a tissue microarray, produced as part of the Specialized Programs of Research Excellence initiative. The staining was scored on a composite scale of 0 to 4 by two independent observers, including one pathologist. In the case of disagreement, the decision is deferred to the pathologist. Each tumor is represented in triplicate. Tumors that scored 2 or better are considered to be positive.

Agar colony formation, cell growth, and apoptosis assays. Transfected cells were plated at a density of 5,000 cells per plate using 35 mm Petri dishes and suspended in 0.4% agar containing 10% FCS RPMI and 50 μg/mL of G418 selective antibiotic, over a 0.8% base agar. The plates were incubated at 37°C and 5% CO2 in a humidified chamber for 14 days. The colonies were counted on the Omnicon Tumor Colony Analyzer.

Changes in proliferation were determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay (23). The experiments were repeated multiple times with similar results. Apoptosis was measured following 72 hours of serum starvation using Apo-BrdUrd kit (Phoenix Flow Systems, San Diego, CA).

Quantitative real-time reverse transcription-PCR. Total RNA was isolated using Trizol (Invitrogen), and quantitative real-time reverse transcription-PCR was carried out using iCycler thermocycler (Bio-Rad, Hercules, CA) and the Quantitect SYBR green reverse transcription-PCR kit (Qiagen, Valencia, CA). PCR was done at the annealing temperature of 59°C with the following primers for β-actin: 5’-TCTCTTCTGGCCATGGAGT-3’ for sense and 5’-TTCTGGATCTCTGGCAATG-3’ for antisense. For MKP1, the annealing temperature of 60°C with the following primers: 5’-CAGT-CTACGATCGGCTGGC-3’ for sense and 5’-GACATTGTCGAGCGTT-GA-3’ for antisense. The expression levels of the transcripts were calculated using the linear exponential phase of amplification throughout 10 to 35 cycles, and each reaction was normalized versus the β-actin transcript internal control.

Results

Notch3 is frequently overexpressed in human lung cancers. The expression frequency of a potential oncogene determines its potential as a target for therapeutics. We have previously shown that Notch3 overexpression is seen in a subset of lung cancer cell lines, suggesting that Notch3 activation is a common event in lung cancer (20). To determine the frequency of Notch3 overexpression in resected, human lung tumors, we probed lung cancer tissue microarrays with an antibody recognizing an epitope in the extracellular domain of Notch3 (24). Tumors that scored 2+ or greater on a scale of 0 to 4 in any of the three triplicate specimens were considered to be positive. Of the 207 evaluable, resected tumors, 39% were positive for Notch3 expression (Table 1). Notch3 is expressed in all the tumor types we surveyed, with squamous histology having the highest frequency at 45%. Differential expression of Notch3 in expression microarray analyses between lung tumors, particularly squamous histologic subtype and normal lung tissue, further supports our observation (25). Both membranous and cytoplasmic staining patterns were observed, similar to that observed with EGFR (Fig. 1).

Complex crosstalk between the Ras and Notch signaling pathway has been extensively shown in cell fate determination, and because the EGF and Ras pathways play prominent roles in tumor growth and survival, we looked for an association between Notch3 and EGF receptor expression (26–30). The scoring system for EGFR expression is stated above. Using χ2, Notch3 expression is found to correlate positively with total EGF receptor expression (P ≤ 0.01), suggesting, but not indicative, of a cooperative interaction between both pathways in the process of lung tumorigenesis (Table 2). Conversely, the correlation can also suggest a negative feedback as a response to elevated Notch3 expression. However, our data below

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support a cooperative relationship rather than an antagonistic relationship. Notch3 was not correlated with overall survival, histology, or staging in our group of patients. This finding indicates that whereas Notch3 may play prominently in the biology of lung tumor, there is no role for Notch3 as a biomarker.

**Notch3 inhibition increases apoptosis and growth factor dependence.** To better understand the biology of Notch3 in tumorigenesis, we asked whether Notch3 is required for the maintenance of the neoplastic phenotype. We created a dominant-negative Notch3 construct based on previous functional studies of Notch receptor deletions (31). This construct contains the extracellular, ligand-binding region without the signaling, intracellular domain (Fig. 2A). To verify that these constructs expressed stable proteins of the anticipated size, expression plasmids were transiently transfected into 293 T cells. Western blot analysis of lysates from transfected 293 T cells showed expression of the predicted size proteins (Fig. 2B). Activation of CBF1-dependent transcription is considered to be characteristic of the Notch signaling pathway activation. Activated genes include basic helix-loop-helix (bHLH) transcription factors such as the hairy-and-enhancer of split (HES) and HES-related proteins (HRT-1; refs. 32, 33). Whereas HES proteins are known to be induced by Notch1, Notch3 is a strong inducer of HRT protein expression (12, 34). To show the functionality of our constructs, we showed that the HRT-1 mRNA level is increased after transfection of the dominant-activating construct and decreased when a Notch3 overexpressing cell line is transfected with a dominant-negative construct (Fig. 2D). On the other hand, no change in HES-1 protein expression was noted, supporting previous studies that Notch3, unlike Notch1, is not a strong inducer of HES genes expression (Fig. 2C; ref. 12).

We stably transfected the dominant-negative Notch3 into the Notch3-overexpressing cell lines, HCC2429 and H460. Tumor cells expressing the dominant-negative construct formed significantly fewer colonies in soft agar compared with vector controls (Fig. 3A, a and b, respectively). In general, the number and size of colonies were also markedly smaller when compared to vector controls. There was an approximately 4-fold reduction in the number of colonies when the Notch3-overexpressing cell lines, HCC2429 and H460, were transfected with the dominant-negative construct. Unlike normal cells, transformed cells often grow in the monolayer culture with little or no serum, a fact attributed to constitutively activated growth pathways or autocrine growth factor loops in cancer cells (35). The growth rate of HCC2429 transfected with the dominant-negative construct is markedly decreased compared with vector controls when the cells are maintained in serum-free medium. However, in the presence of 1% serum, the growth rate of cells transfected with the dominant-negative construct is equivalent to vector controls (Fig. 3B). This finding suggests that activated Notch3 renders tumor cells less dependent on exogenous growth factors.

Increased cell death in the Notch3 dominant-negative transfected observed in serum-free medium suggests that Notch3 may play a role in apoptosis. One pathway known to be important in apoptosis inhibition in cancer cells is the phosphatidylinositol-3-kinase/Akt pathway. After 72 hours in serum-free medium, we observed a 4-fold increase in the number of apoptotic cells (Fig. 3C) associated with dramatically down-regulated levels of phospho-Akt (Fig. 3D).

### Table 1. Expression of Notch3 and EGFR by immunohistochemistry using microtissue arrays

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>n</th>
<th>Notch3+</th>
<th>EGFR+</th>
</tr>
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<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>87</td>
<td>32 (37%)</td>
<td>69 (79%)</td>
</tr>
<tr>
<td>Neuroendocrine</td>
<td>10</td>
<td>2 (20%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Carcinoid</td>
<td>7</td>
<td>1 (14%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>Large cell</td>
<td>4</td>
<td>1 (25%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Small cell</td>
<td>88</td>
<td>40 (45%)</td>
<td>81 (92%)</td>
</tr>
<tr>
<td>Squamous cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinomas</td>
<td>11</td>
<td>4 (36%)</td>
<td>8 (73%)</td>
</tr>
<tr>
<td>Total</td>
<td>207</td>
<td>80 (39%)</td>
<td>168 (81%)</td>
</tr>
</tbody>
</table>

Figure 1. Notch3 is overexpressed in a subset of resected lung cancers and is correlated with EGFR expression. Expression of Notch3 was detected using the antibody 4E1 that recognizes the extracellular domain of the receptor (A, C, E, and G). Comparable tumors stained with a total EGFR antibody (B, D, F, and H). A and B, representative squamous cell carcinoma showing membranous staining of Notch3 and EGFR. In other cases (for example C and D), an adenocarcinoma shows a more cytoplasmic distribution. In a rare lung tumor (E and F), Notch3 expression is detected in an EGFR-tumor. A carcinoid tumor (G and H) fails to show any Notch3 expression, whereas the EGFR staining is strongly positive.
Notch3 induces mitogen-activated protein kinase phosphorylation and down-regulated MKP-1. We noted that in our tumor tissue array data, there is a high degree of correlation between tumors that express the EGF receptor and those that express Notch3. Notch is known to regulate signaling in the EGF pathway and activation of MAPK in Drosophila and Caenorhabditis elegans. Because the MAPK pathway, in particular, the extracellular signal-regulated kinase (ERK) subfamily, plays a prominent role in cellular responses to growth factors and is often altered in cancer, we examined whether Notch3 signaling affects ERK signaling in lung cancer. Under stimulated and unstimulated conditions, the dominant-negative clone transfectants showed a lower level of ERK phosphorylation than that seen in the vector control transfectants. Conversely, higher levels of ERK phosphorylation were noted when Notch3 was activated using the dominant-active form of Notch3 (Fig. 4A).

MAPK activation requires phosphorylation on a threonine and a tyrosine residue within the activation domain. Phosphorylation is usually transient even in the continued presence of activating stimuli, indicating that protein phosphatases represent an important mechanism for MAPK control. Down-regulation of MKPs or resistance to dephosphorylation by MKPs is often observed in human tumors (36–38). Recent data show that Notch antagonizes EGF in developing C. elegans, and one mechanism is through up-regulation of LIP-1, a homologue of mammalian MAPK phosphatases (28). Because Drosophila and C. elegans Notch have the highest homology to human Notch1, and in some systems, Notch3 seems to antagonize Notch1, we hypothesized that Notch3 suppresses MAPK phosphatase expression in cancer cells. Based on these findings, we explored whether one mechanism of ERK regulation by Notch3 is via transcriptional regulation of MKPs. The regulation of MKP1 seems to be one mechanism of delayed MAPK down-regulation by Notch3. However, Notch3 also modulated MAPK phosphorylation acutely following serum induction. Higher levels of phosphorylation of MEK in the Notch3-activated clone following induction with EGF and the total inhibition of MEK activation following treatment with AG1478, an EGF tyrosine kinase inhibitor, suggested that Notch3 modulates not only the MAPK pathway via the regulation of mRNA of phosphatases, but also the tyrosine kinase receptor itself (Fig. 4B).

Inhibition of Notch3 decreases sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors. We have shown that the inhibition of Notch3 signaling increases the dependency of lung cancer cell lines on exogenous growth factors. To examine the potential interaction of the EGF and Notch3 pathways in the maintenance of neoplasia, we examined the effect of modulation of the Notch3 pathway on the sensitivity of lung cancer cell lines to AG1478, a specific EGFR tyrosine kinase inhibitor. When HCC2429 was maintained in EGF-supplemented media and treated with increasing doses of AG1478, the clones transfected with the dominant-negative construct showed a 40-fold increased sensitivity to EGFR inhibitors (Fig. 4D). The IC_{50} was reduced to 0.2 from 8.3 μmol/L. In H460, a lung cancer cell line that has lower Notch3 expression (data not shown) and is more resistant to AG1478 with the IC_{50} of 23.8 μmol/L, the inhibition of the Notch3 pathway also decreases the lung cancer cell line approximately 2-fold to 12.1 μmol/L. Our data provide evidence that Notch3 activation may decrease a tumor’s dependence on the EGF pathway and thus decrease sensitivity to EGFR tyrosine kinase inhibitors.

### Discussion

In our previous study, we found aberrant Notch3 expression to be associated with a translocation in a human lung cancer, as well as overexpression in lung cancer cell lines (20). We also showed that the

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**Table 2. Correlation between Notch3 and total EGFR expressions**

<table>
<thead>
<tr>
<th></th>
<th>EGFR+</th>
<th>EGFR−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch3+</td>
<td>73</td>
<td>7</td>
<td>80</td>
</tr>
<tr>
<td>Notch3−</td>
<td>95</td>
<td>32</td>
<td>127</td>
</tr>
<tr>
<td>Total</td>
<td>168</td>
<td>39</td>
<td>207</td>
</tr>
</tbody>
</table>

**NOTE:** χ², 8.68271; P < 0.01.
ectopic expression of activated Notch3 in the developing lungs of transgenic mice inhibits terminal differentiation of developing pneumocytes (13). These combined data support the hypothesis that the deregulation of Notch3 plays a role in the transformation or maintenance of the neoplastic phenotype. Although Notch3 has been shown to induce lymphoblastic leukemia in transgenic mice and to be negatively correlated with remission in a subset of patients with acute lymphoblastic leukemia, this is the first study to examine the frequency of overexpression and functional role of Notch3 in human lung cancers.

Notch3 plays a role in vascular development and its expression is largely confined to vascular smooth muscle cells in adulthood (22). The prevalence of Notch3 protein expression in our study was 39%, a frequency higher than several other potential oncogenes, such as Bcl2, c-erbB2, or mutated k-ras in lung cancers (39–41). Notch3 does not seem to be a powerful biomarker, as its expression does not correlate with survival in unselected resected lung cancers. We observed that introduction of activated Notch3 alone is not sufficient for the full malignant transformation of the SV40 large tumor antigen-immortalized bronchial epithelial cells (BEAS-2B), suggesting that unlike mutated k-ras, Notch3 is not a potent oncogene (42). However, neoplastic transformation in human epithelial cells is complex, and lesions that alter external growth regulatory signals are undoubtedly important for transformation. Often, the accumulation of multiple genetic alterations are required for full malignant transformation (43, 44). Regardless, the loss of tumorigenicity when the Notch3 pathway is inhibited suggests that Notch3 is important in the survival of the transformed cells in a subset of lung carcinomas.

In development, complex crosstalk between Notch and the ras/MAPK pathway is well established in both invertebrates and vertebrates. We found that Notch3 expression positively correlates with EGFR expression. One prominent mechanism of ERK regulation is the induction of dual specificity MAPK phosphatases (MKP), such as MKP-1. In the majority of mammalian systems, prolonged exposure to growth factors and activation of the p44/p42 cascade induces the expression of MAPK phosphatases-1 and -2 (MKP-1/2) as part of a negative feedback loop, and result in the down-regulation of the MAPK pathway (45–48). Sustained activity of ERK1/2 (p44/p42) is associated with many cancers, and our data suggest that sustained activation of the MAPK pathway is partially accomplished through Notch3 suppression of MKP1 transcription.

Figure 3. Inhibition of the Notch3 signaling pathway mitigates neoplastic phenotypes. A, inhibition of the Notch3 signaling pathway markedly reduces the size of the colonies formed in soft agar (b), compared with vector controls (a) in HCC2429 and H460. The number of colonies formed as measured using the Omnicion Tumor Colony Analyzer is also significantly reduced. B, inhibition of Notch3 increases the dependency of HCC2429 on exogenous growth factors. In serum-starved conditions, the growth of the dominant-negative transfectant is severely inhibited in comparison with that of vector controls. However, with the addition of exogenous growth factors the growth rate is equal to that of vector controls. C, inhibition of Notch3 increases apoptosis in the absence of exogenous growth factors. After 72 hours of exogenous growth factor deprivation, the cell line transfected with the dominant-negative construct shows a higher percentage of apoptosis as measured by Apo-BrdUrd analysis. D, immunoblot detection of phosphorylated Akt protein expression levels in the Notch3 overexpressing cell line HCC2429 stably transfected with vector control (VC) versus the dominant-negative (DN) construct. Compared with the control cell line, the level of phosphorylated Akt is markedly reduced, in particular with serum starvation.

4 Unpublished data.
as well as enhancement of EGFR activation. Thus, our findings show that the Notch3 and EGFR pathway interaction is complex and multisteped.

In summary, we have shown for the first time a role of Notch3 in primary human lung tumors. Whereas Notch3 has been previously implicated in human acute leukemia using constitutively active models, it has not been linked to human epithelial tumors such as lung carcinoma. Our data suggest that Notch3 plays an important role in the maintenance of malignant phenotype, in that inhibition of this pathway dramatically reduces soft agar colony formation, increases apoptosis, and increases the tumor’s dependency on exogenous growth factors. Furthermore, we have data demonstrating Notch3 modulation of EGFR dependency in a human lung cancer cell line naturally overexpressing the gene. From a therapeutic standpoint, Notch3 is a candidate target for therapeutic intervention alone and in combination with growth factor receptor inhibitors. Because about 60% to 80% of lung carcinomas expressed EGFR and far fewer respond to kinase inhibition, our data specifically suggest that combination of Notch3 inhibition with EGFR tyrosine kinase inhibitors may improve clinical response rates (49, 50).

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