

Meningioma Transcript Profiles Reveal Deregulated Notch Signaling Pathway

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Abstract

Meningiomas constitute the second most common central nervous system tumor, and yet relatively little is known about the molecular events that are important for the pathogenesis and malignant progression of these tumors. We have used serial analysis of gene expression to compare the transcriptomes of nonneoplastic meninges and meningiomas of all malignancy grades. A novel finding from this screen is the induction of three components of the Notch signaling pathway: the transcription factor, hairy and enhancer of Split1 (HES1) and two members of the Groucho/transducin-like enhancer of Split family of corepressors, TLE2 and TLE3. TLE corepressors interact and modulate the activity of a wide range of transcriptional regulatory systems, one of which is HES1. We have shown that the transcript and protein levels of HES1, the Notch2 and Notch1 receptors and the Jagged1 ligand are induced in meningiomas of all grades, whereas induction of TLE2 and TLE3 occurs specifically in higher-grade meningiomas. Meningioma cell lines express components of the Notch signaling pathway and an inhibitor of this pathway suppresses meningioma cell survival. These results suggest that deregulated expression of the Notch pathway is a critical event in meningioma pathogenesis and that modulation of this and potentially other signaling pathways by TLE corepressors leads to a more malignant phenotype. (Cancer Res 2005; 65(12): 5070-5)

Introduction

Meningiomas constitute between 13% and 26% of primary brain tumors and are classified into three different WHO grades (1). Higher-grade meningiomas are aggressive and are associated with poor clinical outcomes. Clinically, meningiomas are treated by surgical resection and/or radiation therapy (2). However, meningiomas of all grades can occur in locations that make complete resection impossible and some tumors recur even after surgery and radiation therapy. Mechanisms that cause meningiomas to develop include frequent and early inactivation of the neurofibromatosis 2 and protein 4.1B genes. Mice with biallelic neurofibromatosis 2 deletion in arachnoidal cells form meningiomas (3), and reexpression of both neurofibromatosis 2 and 4.1B in meningioma cells leads to decreased cell proliferation (4). Loss of the G₁-S phase cell cycle checkpoint regulators, CDKN2A and CDKN2B, and p14^{ARF} leads to aggressive meningioma phenotypes (5). Recent studies have implicated loss of tumor suppressor in lung cancer-1 and

induction of ribosomal protein S6 kinase in higher-grade meningiomas (6, 7). These genetic changes, however, do not fully characterize meningiomas. Considerable knowledge is still to be gained about the genes and signaling pathways that are involved in meningioma tumorigenesis.

The purpose of this study was to identify molecular alterations associated with meningiomas. Serial analysis of gene expression (SAGE) is an unbiased, comprehensive gene expression profiling technique (8). Using SAGE, we have identified the induction of components of the Notch signaling pathway in meningiomas. The Notch pathway plays a crucial role in determining cell fate during development and has been implicated in several cancers including the pediatric brain tumor, medulloblastoma, and gliomas (9–11). We show that induction of the Notch receptor, Jagged1 ligand and Notch effector occurs in a proportion of all meningiomas, whereas induction of TLE corepressors is restricted to higher-grade meningiomas. Deregulated Notch signaling represents a potentially new therapeutic target for meningiomas.

Materials and Methods

Tumor and normal tissue samples. All human tissues were collected by the Neurological Surgery Tissue Bank, snap-frozen and stored at -80°C using protocols approved by the University of California, San Francisco, Committee on Human Research. A neuropathologist (A. Bollen) graded each case using the revised 2000 WHO grading system (12). The histologies of frozen meningioma tissue pieces were confirmed by examining adjacent H&E stained sections. Normal controls included autopsy leptomeninges and surgical arachnoid membrane from the cisterna magna. Meningioma cell lines used were IOMM-Lee and immortalized SF3061, SF4068, and SF4433. SF3061 was established from a malignant meningioma, whereas SF4068 and SF4433 were established from benign meningioma tumors. In order for continued growth *in vitro*, SF3061 has been immortalized by expression of telomerase, whereas SF4068 and SF4433 have been immortalized by expression of telomerase and the human papillomavirus E6/E7 genes.

SAGE. Total RNA was isolated using the RNagents kit (Promega, Madison, WI) following the manufacturer's instructions and SAGE libraries were constructed using *Nla*III as the anchoring enzyme and *Bsm*FI as the tagging enzyme as described earlier (13). Plasmid clones (2,304) were sequenced for each library as part of the Cancer Genome Anatomy Project SAGE project. SAGE software v4.0 was used to extract SAGE tags and compare tag frequencies as described previously (13). The meningioma and leptomeninges SAGE library information and tag counts are posted at the Cancer Genome Anatomy Project's SAGE Genie web site (<http://cgap.nci.nih.gov/SAGE>).

Quantitative PCR. Quantitative PCR was done on cDNA templates with the I-cycler machine (Bio-Rad, Hercules, CA) and SYBR Green I (Molecular Probes, Eugene, OR) using PCR conditions and data analysis as described earlier (13). Primers specific for β -actin were used to verify the integrity of the cDNA and to normalize cDNA yields. Primers specific for Notch pathway components were designed to generate 100 to 240 bp products and their sequences are available on request.

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Table 1. Comparison of meningioma and meninges transcript numbers

| SAGE library comparison | Total transcripts compared | Unique transcripts | Altered transcripts* | % differentially expressed |
|-------------------------|----------------------------|--------------------|----------------------|----------------------------|
| Meninges vs. benign 1 | 107,949 | 40,404 | 111 | 0.27 |
| Meninges vs. atypical 1 | 101,921 | 38,387 | 173 | 0.45 |
| Meninges vs. malignant | 115,287 | 41,790 | 152 | 0.36 |
| Benign 1 vs. benign 2 | 104,679 | 38,442 | 87 | 0.22 |
| Benign 1 vs. atypical 2 | 87,136 | 32,315 | 164 | 0.50 |
| Benign 1 vs. malignant | 100,502 | 35,988 | 175 | 0.48 |

*Altered transcripts were derived by performing pair-wise comparisons and counting the number of transcripts that were altered by more than 5-fold and were statistically significant ($P < 0.001$).

Western analysis. The Notch1 (bTAN20), Notch2 (C651.6DbHN) and Jagged1 (TS1.15H) antibodies developed by Spyros Artavanis-Tsakonas were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). The HES1 antibody was a kind gift from Dr. Tetsuo Sudo (Toray Scientific, Japan). The α -tubulin antibody was from Molecular Probes. Total tissue lysates were prepared in radioimmunoprecipitation assay buffer. Protein (75 μ g) was separated by electrophoresis for each sample and was transferred to a

polyvinylidene fluoride membrane. The Notch1, Notch2, and Jagged1 antibody-containing lysates (1:5 dilution) were incubated with the membrane for 30 minutes, followed by incubation with horseradish peroxidase-conjugated goat anti-rat immunoglobulin (Jackson Immunoresearch, West Grove, PA). Bound antibody was visualized by chemiluminescence using the SuperSignal West Pico substrate (Pierce Chemical Co., Rockford, IL). The molecular weights were determined with the use of prestained protein ladders.

Table 2. A subset of the genes induced in atypical and malignant meningiomas

| SAGE tag sequence | Gene symbol (name)* | Accession no. [†] | Fold increase [‡] |
|--|---|----------------------------|------------------------------|
| Cell cycle regulators[§] | | | |
| AAAGTCTAGA | <i>CCND1</i> (cyclin D1) | NM_053056 | 7 \times |
| ACATTTCCAA | <i>G0/S2</i> (putative lymphocyte G ₀ /G ₁ switch gene) | NM_015714 | 18 \times |
| Cell adhesion and invasion | | | |
| AAGGGGCAA | <i>ITGB4</i> (integrin, β 4) | NM_000213 | 10 \times |
| GGGTAGGGTG | <i>ITGA7</i> (integrin, α 7) | NM_002206 | 16 \times |
| CTGGGAGGAG | <i>TM4SF7</i> (transmembrane superfamily 4 member 7) | NM_003271 | 6 \times |
| GCCGGGTGGG | <i>BSG</i> (Basigin) | NM_001728 | 16 \times |
| Glycolysis and hypoxia | | | |
| GCGACCGTCA | <i>ALDOA</i> (aldolase A, fructose-bisphosphate) | NM_000034 | 5 \times |
| TGGCCCCACC | <i>PKM2</i> (pyruvate kinase, muscle) | NM_002654 | 5 \times |
| Apoptosis | | | |
| GGGGGTGGAT | <i>FASTK</i> (Fas-activated serine/threonine kinase) | NM_006712 | 6 \times |
| ACTGTAGAGT | <i>BcoR</i> (Bcl6 interacting corepressor) | NM_017745 | 10 \times |
| TGCCAGGACA | <i>BOK</i> (Bcl2-related ovarian killer) | NM_032515 | 12 \times |
| Signal transduction and transcription factors | | | |
| AGGAAGGAAC | <i>ERBB2</i> (v-erb-b2 avian erythroblastic leukemia viral oncogene) | NM_004448 | 7 \times |
| TATGAGGGTA | <i>RGS5</i> (regulator of G protein signaling 5) | NM_003617 | 22 \times |
| CACTATATT | <i>HES1</i> (hairy and enhancer of Split1) | NM_005524 | 9\times |
| TGGGGGCCGA | <i>TLE2</i> (transducin-like enhancer of Split2) | NM_003260 | 14\times |
| GGATGTGGAG | <i>TLE3</i> (transducin-like enhancer of Split3) | AB046767 | 5\times |
| TGGCCCTCCA | <i>STAT6</i> (signal transducer and activator of transcription 6) | NM_003153 | 8 \times |

NOTE: A few representative genes from each functional class that reflects a known phenotypic difference between benign and higher-grade meningiomas are shown. The meningioma SAGE libraries are posted at <http://cgap.nci.nih.gov/SAGE>. Components of the Notch signaling pathway that were induced in meningiomas are shown in boldface.

*HUGO/GDB nomenclature committee approved symbols for individual genes or are underlined if not yet available. Each gene sequence had a poly-A signal and/or poly-A tail and was matched to the SAGE tag.

[†]The Genbank/RefSeq accession number that was used to identify the gene and contains the differentially expressed tag.

[‡]The fold increase is the ratio of SAGE tags in one of the three higher-grade meningiomas to nonneoplastic leptomeninges.

[§]Genes with potentially similar functions were grouped based on a review of publications and databases.

Immunohistochemistry. Immunohistochemical staining was done on 5 μ m formalin-fixed, paraffin-embedded tissue sections for TLE3 using the M-201 antibody (0.4 μ g/mL, Santa Cruz Biotechnology, Santa Cruz, CA) and for HES1 using the antibody from Dr. T. Sudo (1 μ g/mL). The slides were deparaffinized, rehydrated and sections were sequentially incubated with horse serum, primary antibody, biotinylated secondary antibody, and streptavidin-conjugated horseradish peroxidase (Supersensitive Detection System, Biogenex, San Ramon, CA). Bound antibody was detected using 3,3'-diaminobenzidine and permanently mounted.

Inhibitor assay. Stock solutions of γ -secretase inhibitor I (carboxybenzoyl leucine leucine norleucine; EMD Biosciences, San Diego, CA) were prepared in DMSO. Meningioma cell lines were treated with the indicated concentrations of this inhibitor for 24 hours. Control cells were treated with an equal volume of DMSO.

Colony-forming efficiency assay. The colony-forming efficiency assay was used as described earlier with minor modifications (14). Heavily X-irradiated (50 Gy) IOMM-Lee cells (3.5×10^4) were used as feeder cells and were plated 24 hours in advance of seeding the experimental cells. The plates were incubated for 2 weeks before the colonies were stained with methylene blue. Colonies containing at least 50 cells were counted.

Results

SAGE analysis. To understand the molecular alterations associated with meningiomas, we have compared the transcriptomes of leptomeninges and meningiomas using SAGE. Six SAGE libraries were constructed from leptomeninges, two grade 1 (benign), two grade 2 (atypical), and one grade 3 (malignant) meningiomas. A total of 13,824 clones were sequenced in collaboration with the Cancer Genome Anatomy Project. We extracted 299,298 SAGE tags from the raw sequence data that clustered into 45,774 independent transcripts. We did pair-wise comparisons and Monte Carlo analysis between our SAGE libraries to identify genes that had statistically significant differences in transcript levels. As expected, the transcriptomes of tumors within a particular malignancy grade were more similar to each other when compared with tumors of different malignancy grades. Thus, whereas only 0.22% of the genes were differentially expressed between the two benign tumors, 0.45% to 0.5% of the genes were differentially expressed in the benign to higher-grade tumors

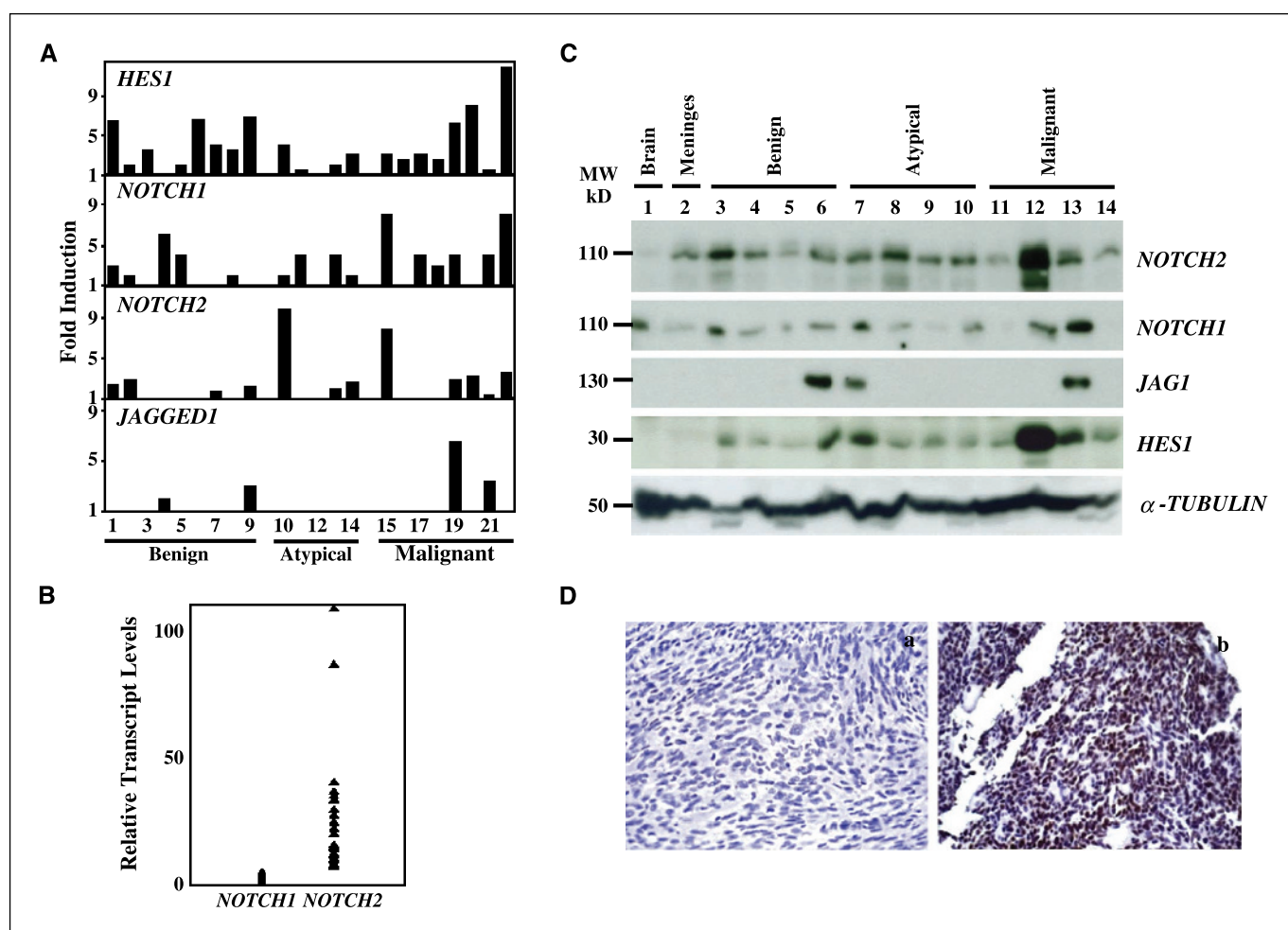


Figure 1. Deregulated expression of Notch pathway components in meningiomas. **A**, transcript abundance of HES1, Notch2, Notch1, and Jagged1 in nine benign (lanes 1-9), five atypical (lanes 10-14), and eight malignant (lanes 15-22) primary meningioma tumors were evaluated using quantitative PCR. Two nonneoplastic meninges samples were used to establish control baseline levels. Fold induction, ratio of the transcript levels in the tumor to the average transcript levels in the two nonneoplastic meninges samples. The histological subtypes are meningothelial (lanes 1, 3, 5, and 7), transitional (lanes 2, 6, 8, and 9), angiomatous (lane 4), atypical (lanes 10-14), and malignant (lanes 15-22). **B**, relative transcript levels of Notch1 and Notch2 in individual meningioma tumors (\blacktriangle) are plotted showing the significantly higher levels of Notch2. **C**, protein levels of Notch1, Notch2, Jagged1, and HES1 in normal brain (lane 1), leptomeninges (lane 2), four benign (lanes 3-6), four atypical (lanes 7-10), and four malignant (lanes 11-14) meningiomas were measured by Western blot analysis on total cell lysates. α -Tubulin was included as a loading control. The histological subtypes are transitional (lanes 3-6), atypical (lanes 7-10), and malignant (lanes 11-14). **D**, *in vivo* expression of HES1 in primary meningioma tumors. Immunohistochemistry was used to distinguish between HES1-negative (a) and HES1-positive meningiomas (b). HES1 staining is nuclear. Original magnification is $\times 400$.

(Table 1). Also, benign tumors were more similar to nonneoplastic meninges when compared with atypical and malignant tumors.

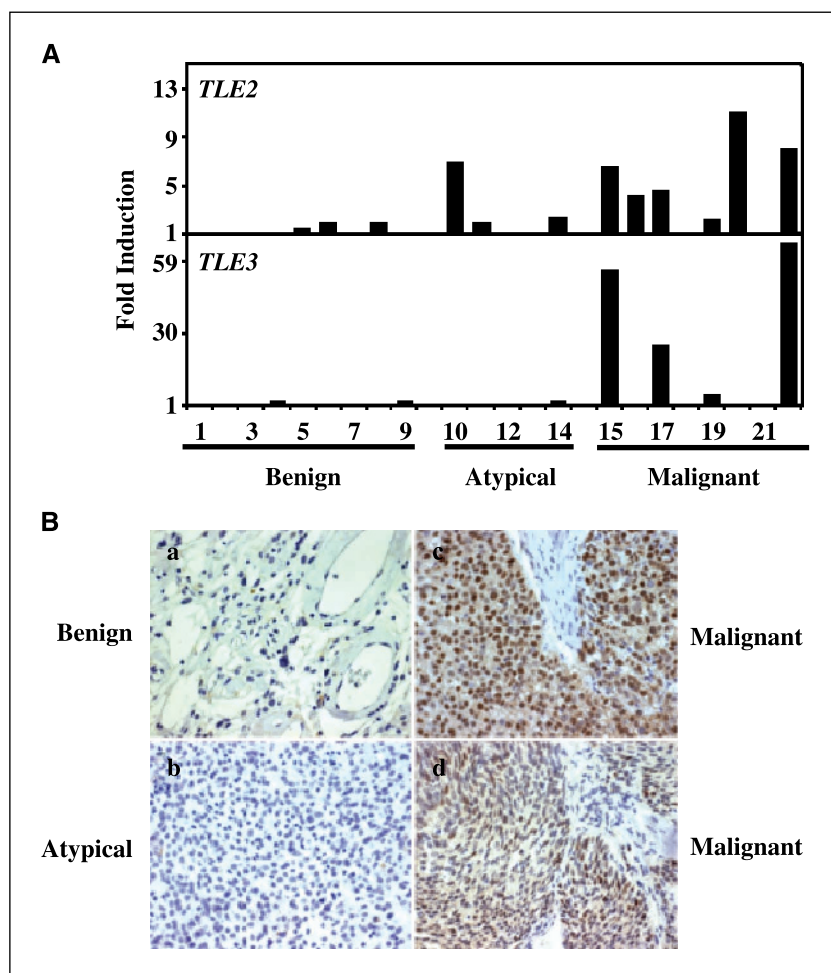
We focused our initial analysis on genes that were induced in the high-grade meningiomas because this population is expected to contain candidate tumor markers, oncogenes, and components of activated signal transduction pathways. We identified 165 genes that are induced by at least 5-fold ($P < 0.001$) in at least one of the three higher-grade meningiomas when compared with leptomeninges. Additionally, these genes were induced by at least 2-fold when compared with both benign libraries. Functional classification of these differentially expressed genes identified patterns of altered expression in high-grade meningiomas (Table 2). There was an alteration in the gene expression of several cell cycle and apoptosis regulators, reflecting the increased cell proliferation rates in higher-grade meningiomas. Regional necrosis and enhanced invasion of normal brain are features that distinguish malignant meningiomas from the benign forms. Consistent with the histopathology, we observed increased transcript levels of several glycolytic enzymes and genes that potentially contribute to invasion (Table 2). A novel finding from this screen is the induction of three members of the Notch signaling pathway: the nuclear basic helix-loop-helix protein, *HES1* and two members of the transducin-like enhancer of Split family of transcriptional corepressors, *TLE2* and *TLE3*.

Deregulated notch signaling in meningiomas. Given our preliminary findings, we used reverse transcription followed by

quantitative PCR as an independent technique to assess the transcript levels of *HES1* in nine benign, five atypical, and eight malignant meningiomas in comparison with nonneoplastic meninges. *HES1* transcript levels were induced by >3-fold in six of nine benign, one of five atypical, and three of eight malignant meningiomas (Fig. 1A). Thus, a survey of a larger number of meningiomas showed that induction of *HES1*, the Notch effector, occurs in meningiomas of all grades, implying that it is an early change in the malignant progression of meningiomas.

To investigate the mechanism of induction of *HES1*, we looked at upstream components of the Notch pathway. Mammalian systems express four different Notch homologues and five different Notch ligands, although there is no information available about which ones are expressed in meninges or meningiomas. This led us to perform RT-PCR using primers specific for the four Notch homologues and the two members of the Jagged family of ligands. Notch2 was the main receptor and Jagged1 was the main ligand homologue expressed in meninges and meningiomas (data not shown). The Notch1 homologue was also expressed, but at much lower levels. The transcript abundance of Notch2, Notch1, and Jagged1 was assessed by quantitative PCR in the same panel of meningioma samples used above for *HES1*. Once again, Notch1 transcript levels were considerably lower than Notch2 transcript levels (Fig. 1B). However, both Notch1 and Notch2 transcript levels were elevated in a proportion of meningiomas of all three grades (Fig. 1A). Jagged1 was also induced in meningiomas. However,

Figure 2. Expression of TLE corepressors in meningiomas. **A**, fold induction of transcript levels of *TLE2* and *TLE3* in nine benign (lanes 1-9), five atypical (lanes 10-14), and eight malignant (lanes 15-22) primary meningioma tumors were calculated compared to nonneoplastic meninges using quantitative PCR. The cases are identical to the ones used in Fig. 1A. Note that the scale of the fold induction differs for the *TLE2* and *TLE3* graphs. **B**, *in vivo* expression of *TLE3* in primary meningioma tumors. Immunohistochemistry with a polyclonal antibody specific to *TLE3* (Santa Cruz Biotechnology) was used to show that some benign (a) and atypical (b) meningiomas were negative for *TLE3*. A subset of malignant meningiomas (c and d) showed strong nuclear positivity for *TLE3*. Original magnification is $\times 400$.



transcript levels of Notch and Jagged1 did not correlate well with those of *HES1*. This is not surprising because activation of the Notch receptor is posttranscriptionally regulated.

We extended our expression analysis to the protein level using Western blot analysis on a panel of four benign, four atypical, and four malignant meningiomas (Fig. 1C). Monoclonal antibodies specific for Notch1 and Notch2 recognize the intracellular domain of the Notch receptor heterodimer (15). At the protein level, meninges expressed higher levels of Notch2 when compared with Notch1 (Fig. 1C, lane 2). This is in contrast to normal brain where Notch1 is expressed at higher levels (Fig. 1C, lane 1). Both the Notch2 and Notch1 receptors were overexpressed in some meningioma tumors when compared with normal meninges. Additionally, 3 of 12 meningiomas had higher levels of Jagged1 when compared with normal meninges. Protein levels of *HES1* correlated well with the protein levels of Notch and Jagged. For example, the benign meningioma #6 had high levels of Jagged1 and an equivalently high level of *HES1*, and malignant meningioma #12 had high levels of Notch2 and high levels of *HES1* (Fig. 1C). We have also used immunohistochemistry to show that *HES1* protein was correctly localized to the nucleus in *HES1*-positive meningiomas (Fig. 1D). These results confirm that the Notch pathway is deregulated in meningiomas of all three grades and that induction of *HES1* could be a common consequence of up-regulation of both the Notch receptors and Jagged ligand.

TLE induction in higher-grade meningiomas. Quantitative PCR analysis revealed that induction of *TLE2* and *TLE3* was seen only in higher-grade meningiomas (Fig. 2A). *TLE2* was induced by >3-fold in zero of nine benign, one of five atypical and five of eight malignant tumors. Overexpression of *TLE3* was observed in 50% of malignant meningiomas with the amplitude of induction ranging from 4- to 65-fold. Using immunohistochemistry, we have shown that protein levels of *TLE3* are induced in some malignant meningiomas when compared with benign and atypical meningiomas (Fig. 2B). Importantly, *TLE3* is correctly localized to the nucleus, where it can carry out its transcriptional corepressor functions.

Inhibitor assay. We evaluated the expression of Notch pathway components in four meningioma cell lines. Meningioma cell lines expressed similar levels of Notch2 but some meningioma cell lines had significantly elevated levels of *HES1* (Fig. 3A and B). One potential reason for this could be the elevated expression levels of Jagged1 that correlated well with the levels of *HES1*. To evaluate the consequence of Notch pathway deregulation on meningioma growth, we used an inhibitor of the γ -secretase protease complex that cleaves and activates the Notch receptor. The SF4433 cell line was treated with 0 to 5 $\mu\text{mol/L}$ of γ -secretase inhibitor I for 24 hours and the effect of this inhibitor on cell growth and survival was evaluated using the colony-forming efficiency assay (Fig. 3C). Complete inhibition was observed by 5 $\mu\text{mol/L}$ with an IC_{50} of 1.5 $\mu\text{mol/L}$. Similar results were observed with SF4068 (data not shown).

Discussion

Analysis of global gene expression profiles provides insights into the molecular basis of neoplasia. We have used SAGE to identify deregulation of the Notch signaling pathway in meningiomas. Although it is clear that Notch is involved in the genesis of diverse tumor types, the nature of Notch function in cancer is complex. In T cell leukemias and breast cancers, Notch is an oncogene-

promoting tumorigenesis, whereas in other cancers, like basal cell carcinomas, Notch behaves as a tumor suppressor (16, 17). In yet other cancers, like cervical cancers, Notch pathway components are high in low-grade tumors, whereas more aggressive cervical cancers express low levels of the same components (18). We have investigated the frequency and mechanism of deregulation of the

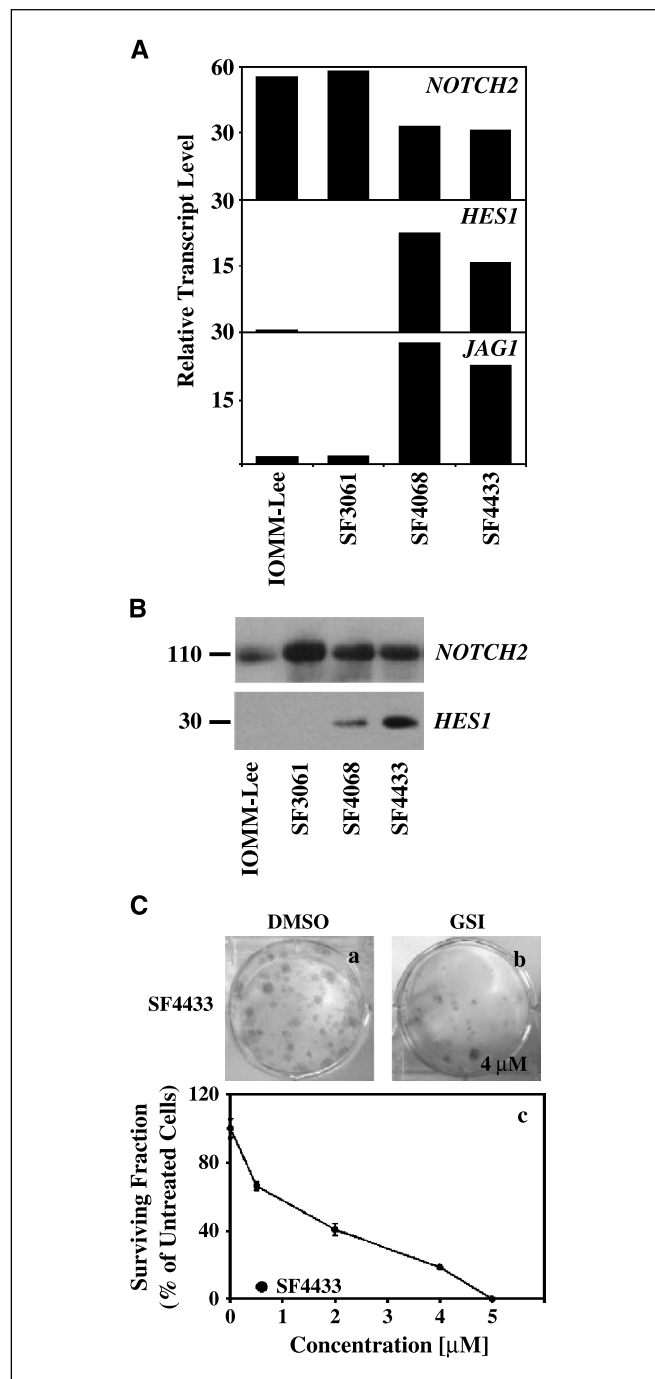


Figure 3. Effect of inhibition of γ -secretase on meningioma cell lines. A, quantitative PCR of Notch2, JAG1, and *HES1* in four meningioma cell lines, IOMM-Lee, SF3061, SF4068, and SF4433. B, Western blot analysis of Notch2 and *HES1* in meningioma cell lines. C, representative plates from the colony-forming efficiency assay for SF4433 (a and b) treated with DMSO (a) and with 4 $\mu\text{mol/L}$ (b) γ -secretase inhibitor I. The surviving fraction from the colony-forming efficiency assay are plotted against the concentration of γ -secretase inhibitor I (c).

Notch pathway in meningiomas. Forty-five percent of meningiomas of all grades have induction of HES1, suggesting that deregulation of Notch signaling is a frequent, early, and critical genetic alteration in meningioma pathogenesis. Induction of HES1 correlates well with overexpression of the Notch receptor or the Jagged ligand, implying that Notch activation in meningiomas could be achieved by either ligand-mediated and/or ligand-independent mechanisms. Ligand-mediated Notch activation is observed in Hodgkin's and anaplastic large cell lymphomas, whereas ligand-independent activation is seen in leukemias and breast cancers (19–21).

Nonneoplastic meninges and meningiomas express significantly lower levels of Notch1 compared with Notch2. However, both Notch1 and Notch2 are induced in meningiomas, suggesting that either or both these homologues contribute to meningioma tumorigenesis. This is in contrast to medulloblastomas, where Notch2 levels are induced and Notch1 levels are unaffected (9). In these tumors, Notch2 promotes tumor growth, whereas Notch1 has the opposite effect of inhibiting tumor growth. Whether the Notch1 and Notch2 receptors have redundant or opposing functions in the pathogenesis of meningiomas awaits a detailed functional analysis. In gliomas, Notch1 and its ligands, Delta-like-1 and Jagged1, are overexpressed (11). Inhibition of Delta-like-1 induces glioma cell apoptosis (11), exemplifying the important role of Notch ligands in cancer and suggesting that Jagged1 induction probably has important functional consequences in meningiomas.

The TLE corepressors have no intrinsic DNA binding activity but interact with a wide variety of transcription factors, converting them from activators to repressors (22). TLE1 is thought to contribute to the development of more aggressive forms of hepatocellular carcinomas and induces anchorage-independent growth in chicken embryo fibroblasts (23, 24). Our results suggest that TLE1 homologues, TLE2 and TLE3, are involved in the

formation of aggressive meningiomas. TLE3 is induced and correctly localized to the nucleus of malignant meningiomas. TLE corepressors function by modulating the activity of HES1 (25), suggesting that interactions between HES1 and TLE may induce an alternate set of transcriptional targets resulting in more aggressive meningiomas. Alternatively, TLEs could be modulating the activity of other, as yet unidentified, signaling pathways.

γ -Secretase cleaves and activates the Notch receptor and inhibitors of this enzyme could be potential therapeutic targets for meningiomas (26). We show that γ -secretase inhibitor I decreased survival of meningioma cell lines. As much as 10 $\mu\text{mol/L}$ γ -secretase inhibitor I has been used on melanocytes with no toxicity (27). γ -Secretase has numerous substrates, like CD44 and syndecan, in addition to Notch and γ -secretase inhibitor I could have additional targets besides γ -secretase (28). Further studies are needed to dissect the mechanism of action of γ -secretase inhibitor I on meningioma cell lines. In conclusion, our data identifies a signal transduction pathway that is commonly deregulated in meningiomas and represents a potential new therapeutic target.

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