SLIT2, a Human Homologue of the Drosophila Slit2 Gene, Has Tumor Suppressor Activity and Is Frequently Inactivated in Lung and Breast Cancers¹

Ashraf Dallol, Nancy Fernandes Da Silva, Paolo Viacava, John D. Minna, Ivan Bieche, Eamonn R. Maher, and Farida Latif²

ABSTRACT

Slit2 plays a vital role in axon guidance by signaling through Robo receptors. Recent evidence suggests that Slit2 protein may function in other settings because human and Xenopus Slit2 has been shown to inhibit leukocyte chemotaxis. SLIT2 protein is a putative ligand for the ROBO receptors. We recently demonstrated that ROBO1 is inactivated by promoter region hypermethylation in <20% of human cancers; furthermore, tumor suppressor activity has not been shown. Thus, the importance of ROBO1 inactivation in human cancer is uncertain. Therefore, we investigated the status of SLIT2 located at 4p15.2 in lung and breast cancers. Although somatic SLIT2 mutations were not detected, epigenetic inactivation was common. SLIT2 promoter methylation was detected in 59% of breast cancer, 77% of non-small cell lung cancer, and 55% of small cell lung cancer cell lines. In these tumor lines, SLIT2 expression was restored by treatment with a demethylating agent. SLIT2 promoter methylation was detected in 43% of breast cancer, 53% of non-small cell lung cancer, and 36% of small cell lung cancer primary tumors. The majority of methylated tumors demonstrated allelic loss at 4p15.2. In addition, SLIT2 expression was down-regulated in methylated breast tumors, relative to normal control, as demonstrated by quantitative real-time reverse transcription-PCR. Overexpression of SLIT2 suppressed >70% of colony growth in each of three breast tumor lines (with either absent or low SLIT2 expression). Because SLIT2 is primarily a secreted protein, SLIT2-conditioned medium suppressed the growth of several breast cancer lines (with absent or weak SLIT2 expression) by 26–51% but had no significant effect on a breast tumor cell line that expresses normal levels of SLIT2. These findings demonstrate that SLIT2 is frequently inactivated in lung and breast cancer by promoter region hypermethylation and allele loss and is an excellent candidate for the lung and breast tumor suppressor gene previously mapped to 4p15.2.

INTRODUCTION

The Slit family comprises large ECM secreted and membrane-associated glycoproteins. Human Slits (SLIT1, SLIT2, and SLIT3) are candidate ligands for the repulsive guidance receptors, the ROBO gene family. The Slit-Robo interactions mediate the repulsive cues on axons and growth cones during neural development. The Slit2-Robo1 interaction has been shown to be enhanced by cell surface heparan sulfates including glypican-1 (1, 2). The Slit proteins are highly conserved in evolution and contain a putative signal peptide, four tandem arrays of leucine-rich repeats required for the Slit-Robo interaction, and seven to nine EGF repeats, which are conserved in evolution and are thought that different axons have specific responses to particular SLIT2 fragments (3).

SLIT2 is expressed in neural as well as nonneuronal tissues. Rat Slit2 is expressed in lung, breast, kidney, and heart (6). In situ hybridization of human kidneys revealed the expression of SLIT2 in mesangial and epithelial cells in the glomeruli, in epithelial cells in the tubules, and in endothelial cells of both arterioles and venules in the kidney (6). In addition, Unigene cluster Hs.29802 shows that SLIT2 is expressed in adult lung and kidney in addition to heart. This broad pattern of SLIT2 expression suggests that it may have other functions. Slit2 has been shown to inhibit leukocyte chemotaxis (6), possibly through the Slit2 effect on actin cytoskeleton organization mediated through cdc-42 (7). Slit protein triggers Robo-DCC (deleted in colon cancer) interaction with the subsequent loss of responsiveness of DCC to its ligand, netrin-1 (8). This loss may activate apoptotic pathways through caspase 3 and caspase 9 (9). Exon 2 of ROBO1 is homozygously deleted in two lung tumor cell lines and one breast tumor cell line (10). Most mice with deletion of exon 2 of Robo receptor die because of delayed lung maturation, whereas the surviving mice develop bronchial hyperplasia (11).

The SLIT2 gene has been mapped to chromosome 4p15.2 (12). Microsatellite markers from this region show LOH in 63% of mesothelioma, 60% of SCLC, and 25% of NSCLC (13). Around 63% of breast tumors also show LOH at 4p15.1–15.3 (14). Deletion of the 4p15.1–15.3 region has also been shown to occur in colorectal carcinoma (15), invasive cervical cancer (16), head and neck squamous cell carcinoma (17), and bladder cancer (18).

Along with mutation screening of candidate TSGs, methylation analysis of promoter region CpG islands (if present) is required to study tumor-specific inactivation. Aberrant methylation of promoter region CpG islands has been largely associated with gene silencing in human cancers. This has been reported for several TSGs, most notably for the new TSG RASSF1A located at 3p21.3 (19, 20).

We have shown that the putative SLIT2 receptor, DUTT1/ROBO1, is methylated in <20% of breast tumors and clear cell renal cell carcinomas. Methylation of the putative DUTT1 promoter was a rare event in lung carcinomas (21). The coding region of the DUTT1 gene lacked any inactivating mutations, and the gene was expressed in the majority of tumor cell lines examined. However, LOH of DUTT1 occurred frequently in lung, breast, and kidney tumors, prompting us to hypothesize that haploinsufficiency of DUTT1 coupled with haploinsufficiency/inactivation of its ligand could be detrimental for tumor progression and metastasis. Thus ROBO1 remains a candidate for the lung and breast 3p12 TSG (22). We reasoned that if ROBO1 inactivation was a significant event in human breast and lung cancers, then other components of the signaling pathway might also be involved in cancer. Therefore, we undertook genetic, epigenetic, and...
Green unmethylated cytosine residues were sulfonated by incubation in 3.12 M genomic DNA was denatured in 0.3 M NaOH for 15 min at 37° C. The conversion reaction was completed by desulfonating in 0.3 M NaOH for 10 min at room temperature. According to the manufacturer’s instructions. The conversion reaction was performed with the working draft sequence of human genome, the UCSC assembly. Mutation screening of 37 exons was performed on 22 SCLC, 9 NSCLC, and 7 breast cell lines by the PCR-single-strand conformational polymorphism method using intronic primers. Aberrantly migrating bands were sequenced on an ABI377 automated sequencer. The expected product size is 387 bp. 

Materials and Methods

Patients and Samples. DNA from a total of 36 SCLC and 30 NSCLC tumors (6 squamous cell tumors, 11 adenocarcinomas, 3 large cell tumors, 1 adenosquamous tumor, and 9 tumors of unknown histopathology) and corresponding normal tissue and 31 SCLC and 17 NSCLC tumor cell lines was analyzed. The primary tumors and corresponding normal tissue were obtained by either autopsy or operation. A total of 37 invasive ductal breast carcinomas and corresponding normal breast tissue were analyzed. Formalin-fixed, paraffin-embedded sections were obtained as described previously. They were either detected by mammographic screening or had presented symptomatically. None of the tumors were from women with a known family history of breast or other cancers. All tissue histological assessment was performed by P. V. In addition, DNA obtained from an additional 16 breast tumors (frozen tissue) and 27 breast tumor cell lines was also analyzed for SLIT2 methylation.

Bisulfite Modification and Methylation Analysis. Bisulfite DNA sequencing was performed as described previously. Briefly, 0.5–1.0 μg of genomic DNA was denatured in 0.3 M NaOH for 15 min at 37° C, and then unmethylated cytosine residues were sulfonated by incubation in 3.12 M sodium bisulfite (pH 5.0; Sigma)/5 mM hydroquinone (Sigma) in a thermocycler (Hybaid) for 20 cycles of 30 s at 99° C and 15 min at 50° C. The sulfonated DNA was recovered using the Wizard DNA cleanup system (Promega) in accordance with the manufacturer’s instructions. The conversion reaction was completed by desulfonating in 0.3 M NaOH for 10 min at room temperature. The DNA was ethanol-precipitated and resuspended in water.

The SLIT2 putative promoter region was predicted by Promoter Inspector software. This region is from −761 to −212 relative to the translation start site. The region was amplified from breast and lung cell lines not expressing SLIT2 or expressing SLIT2 using the primers Sl2MOD4F (5’-GGGAGGT-GGATGTGTAGATTT-3’) and Sl2MOD4R2 (5’-CAAAAAATCCTCTTAAACACATTTTATCTCAAATAA-3’). We used 0.02 volume of the first PCR reaction (with primers Sl2MOD4F and Sl2MOD4R2) in another PCR reaction using Sl2MOD4F and the nested primer, Sl2MOD4R (5’-ACTAAACACTTCCAAACACTAATTAAATACAAAAAA-3’). The PCR conditions for both the first and second PCR were 95° C for 10 min, followed by 30–40 cycles of 1 min of denaturation at 95° C, 1 min of annealing at 54° C, and 2 min of extension at 74° C. The PCR products were cloned into pGEM T-Easy vector, and at least five clones were sequenced. The methylation-sensitive primers were designed based on the sequencing data of the PCR-amplified bisulfite-modified cell line genomic DNA. The methylated alleles were amplified using Sl2-MSP-F (5’-CGGTGAATTCGTCGGCGGAGTCAGGCCC-3’) and Sl2-MSP-R (5’-GGAGAGGACGAAAACCCACCAACAAAAAAGGC-3’). The PCR cycling conditions for this reaction consisted of 10 min at 95° C followed by 30 cycles of 1 min of denaturation at 95° C, 1 min of annealing at 68° C, and 2 min of extension at 74° C. The unmethylated allele was amplified using primers Sl2-UPF-F (5’-TGTTTTAGTTGGGTATGGAAGTGTAGTGGAGGTTGAAGGTG-3’) and Sl2-UPF-R (5’-CACTGGAAGGCCCATCACCACACAC-3’). The PCR cycling conditions were similar to those above, but annealing was done at 59° C.

Cell Lines and 5-aza-dC Treatment. Lung and breast carcinoma cell lines were routinely maintained in RPMI 1640 (Invitrogen, San Diego, CA) supplemented with 10% FCS at 37° C, 5% CO2. The demethylating agent 5-aza-dC (Sigma) was freshly prepared in dd H2O and filter sterilized. Cells (5–10 × 104) were plated in a 25-cm2 flask in RPMI 1640 supplemented with 10% FCS. Forty-four h later, cells were treated with 10 μM 5-aza-dC. The medium was changed 24 h after treatment and then changed every 3 days. RNA was prepared at 10 days after treatment using the RNeasy kit (Qiagen) according the manufacturer’s guidelines. SLIT2 expression was detected by RT-PCR using the primers 5’-GGTGTTCCCTCTGATGAGAAAG-3’ and 5’-GTTTGTAGGAGAACACACTCCTG-3’. The expected product size is 387 bp. Expression of GAPDH was used as a control. The GAPDH primers were 5’-TGAGTCTGCAACGTCAGTGG-3’ and 5’-CTGGGGCGCATGAGTCACCCAC-3’. The expected product size is 982 bp.

Mutation Analysis of Primary Tumors and Cell Lines. Intron-exon boundaries were determined by matching the cDNA sequence for SLIT2 (GenBank accession number AB017168) with the working draft sequence of human genome, the UCSC assembly. Mutation screening of 37 exons was performed on 22 SCLC, 9 NSCLC, and 7 breast cell lines by the PCR-single-strand conformational polymorphism method using intronic primers. Abruptly migrating bands were sequenced on an ABI377 automated sequencer. The sequences of the primers used, along with the annealing temperature and expected product size, are available upon request.

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SLIT2 HAS TUMOR SUPPRESSOR ACTIVITY

Microsatellite Repeat Analysis. PCR amplification of the dinucleotide repeat microsatellite sequence of marker D4S1546 was performed on 36 SCLC, 30 NSCLC, and 37 breast carcinomas. The D4S1546 marker was the nearest possible marker (within 100 kb) available to the SLIT2 gene on 4p15.2 according to GDB and the UCSC assembly of the human genome sequence. The PCR conditions were 96°C for 5 min, followed by the addition of 0.2 unit of Taq polymerase (Invitrogen), followed by 35 cycles of 96°C for 30 s, 52°C for 30 s, and 72°C for 30 s. A final extension step of 5 min at 72°C was performed.

Real-Time RT-PCR. Quantitative real-time RT-PCR was performed as described previously (24). Briefly, cDNA was made from total RNA extracted from frozen normal and tumor breast tissues. We quantified transcripts of the TBP (TATA box-binding protein) gene as the endogenous RNA control. Each sample was normalized on the basis of TBP content. Results, expressed as N-fold differences in target gene expression relative to the TBP gene (terned \(N_{\text{expr}}\)), were determined by the following formula: \(\Delta C_{\text{t, sample}} = \Delta C_{\text{t, control}}\), where the \(\Delta C_{\text{t}}\) value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the TBP gene. The \(N_{\text{expr}}\) values of the samples were subsequently normalized such that the mean ratio of the normal breast samples would equal a value of 1. The nucleotide sequences of the primers used for PCR amplification were as follows: (a) SLIT2-U (5'-CTTGGTGGAAATGTTGCACTAA-3') and SLIT2-L (5'-TTTCGATTTGCTTCACATCAAAGT-3') with a size of 76 bp; and (b) SLIT2-A (5'-TGACAAGGAGCCAGTTGAA-3') and SLIT2-B (5'-CACATACAGCTCCCCACCA-3') with a TBP-specific product size of 132 bp. PCR was performed using the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 15 s and 65°C for 1 min. Experiments were performed with duplicates for each data point.

Antibodies and Western Blotting. SLIT2- or vector only-transfected COS-7 cells or condition media were solubilized in SDS-PAGE sample loading buffer and run out on a SDS-PAGE gel (7.5%). The samples were Western blotted by standard methods with a monoclonal 9E10 anti-myc antibody (Clontech) or anti-SLIT2 antibody (Ref. 5; gift from M. Tessier-Lavigne, Stanford University, Stanford, CA). Total protein levels were quantified using the Bradford assay.

Plasmid Constructs and Growth Suppression Analysis. The SLIT2 expression construct was made by cloning the full-length human Slit2 coding region into the BamHI-XbaI sites of pSecTagB vector (Invitrogen); thus, it contains both myc and His epitope tags at its COOH terminus (5). Transfection with FuGENE 6 reagent (Roche Molecular Biochemicals) used 2 µg of each plasmid per 25 cm² flask containing 5 × 10⁴ cells seeded 24 h before transfection. Forty-eight h after transfection, 5 × 10⁴ transfected cells were seeded and maintained in RPMI 1640 and 10% fetal bovine serum supplemented with 50 µg/ml zeocin (Invitrogen). Surviving colonies were counted 14–21 days later, after staining with crystal violet. SLIT2-conditioned media were prepared by transient transfection of COS-7 cells with SLIT2 or vector only plasmids. Forty-eight h after transfection, the media were cleared by centrifugation, and a total of 10 µl SLIT2 protein was added to 5 × 10⁴ cells seeded 24 h earlier in each well of 6-well plates. After 4 days, the cells in each indicated conditioned medium were counted using a hemocytometer.

RESULTS

SLIT2 Is Silenced by Hypermethylation in Lung and Breast Cancer Cell Lines and Is Down-Regulated in Breast Cancer. We analyzed 15 breast and lung tumor cell lines for SLIT2 expression using RT-PCR and found that the majority (62%) had weak or absent SLIT2 RNA expression (Fig. 3A). We analyzed these cell lines for SLIT2 promoter region hypermethylation (as determined by the presence of a CpG island and analysis of the SLIT2 promoter region hypermethylation (as determined by the presence of a CpG island and analysis of the SLIT2 promoter region, we sequenced at least five alleles for each of the nine cell lines (Fig. 2B).
We also sequenced normal/tumor pairs from breast and lung cancer and found that they exhibited tumor-specific SLIT2 hypermethylation (Fig. 2B). SLIT2 promoter region hypermethylation correlated with expression status, and all cell lines with promoter methylation demonstrated reduced (methylated as well as unmethylated alleles detected) or absent (only methylated alleles detected) mRNA expression. Cell lines with apparently normal SLIT2 mRNA levels had no detectable methylation (Fig. 3A). To establish that methylation was responsible for silencing SLIT2 gene expression, we treated three breast cancer cell lines with SLIT2 methylation with the demethylating agent 5-aza-dC. SLIT2 expression was restored in these lines after 5-aza-dC treatment (Fig. 3B). Using quantitative real-time RT-PCR, we found that SLIT2 expression was significantly down-regulated in breast tumors, in which it is methylated compared with SLIT2 expression in normal breast tissues (Fig. 3C).

**Table 1** Incidence of methylation in breast and lung tumors, cell lines, and nonmalignant control tissues

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total no.</th>
<th>No. methylated (%)</th>
</tr>
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<tbody>
<tr>
<td>Breast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell lines</td>
<td>27</td>
<td>16 (59%)</td>
</tr>
<tr>
<td>Primary tumors</td>
<td>53</td>
<td>23 (43%)</td>
</tr>
<tr>
<td>Nonmalignant breast</td>
<td>37</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>NSCLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell lines</td>
<td>17</td>
<td>13 (77%)</td>
</tr>
<tr>
<td>Primary tumors</td>
<td>30</td>
<td>16 (53%)</td>
</tr>
<tr>
<td>Nonmalignant lung</td>
<td>30</td>
<td>4 (13%)</td>
</tr>
<tr>
<td>SCLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell lines</td>
<td>31</td>
<td>17 (55%)</td>
</tr>
<tr>
<td>Primary tumors</td>
<td>36</td>
<td>13 (36%)</td>
</tr>
<tr>
<td>Nonmalignant lung</td>
<td>36</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>Peripheral blood lymphocytes</td>
<td>20</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

The SLIT2 Gene Is Not Commonly Mutated in Lung and Breast Cancers. We analyzed the 37 coding exons of SLIT2 by PCR-single-strand conformational polymorphism in the primary lung tumors and
breast tumor lines without SLIT2 methylation. We detected five intronic and two exonic polymorphisms and two missense substitutions (W983R in a breast tumor line and H1090G in a SCLC tumor sample). The H1090G missense substitution was also found in the corresponding normal DNA, hence it is a germ-line missense change. The W983R missense change was in a breast tumor cell line, and DNA from corresponding normal tissue was not available; hence, this change could be either somatic or germ line. The W983R and H1090G changes occur in evolutionarily conserved tryptophan and histidine, and both of these amino acids are located in an EGF-like domain.

**SLIT2 Inactivation Is Consistent with Knudson’s Two-hit TSG Hypothesis.** LOH analysis at D4S1546 (which maps within 100 kb of SLIT2) demonstrated allele loss in 28% and 30% of informative lung and breast cancers, respectively (Table 2). SLIT2 methylation was present in 88% (seven of eight) NSCLC tumors, 50% (four of eight) of SCLCs, and 67% (two of three) of breast tumors with allele loss at D4S1546.

**Overexpression of SLIT2 Induces Growth Suppression.** Having demonstrated that epigenetic inactivation of SLIT2 is frequent in two major human cancers, we investigated tumor suppressor activity of SLIT2 in in vitro colony formation assays. A wild-type SLIT2 expression plasmid or the pSecTagB empty vector was transfected into three breast cancer cell lines with absent (HTB-19) or low (T-47D and MCF-7) SLIT2 expression. Both SLIT2 and empty constructs expressed a zeocin resistance gene, but transfection with SLIT2 reduced the number of zeocin-resistant colonies by >70% compared with transfection with empty vector in all three cell lines tested (Fig. 5, A and B). This effect was consistent through five independent experiments and using three independent plasmid DNA preparations. There were no significant differences between the colony formation abilities of the three cell lines tested after SLIT2 transfection (P > 0.05).

**Growth Suppression by Conditioned Medium from COS-7 Cells Transfected with SLIT2.** Because SLIT2 is primarily a secreted ECM protein, we analyzed the effect of conditioned medium harvested from COS-7 cells transfected with SLIT2 on the T-47D, MCF-7, and HTB-19 breast cancer cell lines. SLIT2 expression in COS-7 cells and conditioned media was confirmed by Western blotting (Fig. 6A). The previously reported proteolytic cleavage of SLIT2 was confirmed (4, 5). SLIT2 induced up to 51% growth suppression or cell death compared with conditioned medium from vector control-transfected COS-7 cells (Fig. 6B). This effect was consistent throughout three independent experiments. This effect corresponded to the level of inhibition of colony formation ability in the three different tumor cell lines. To check whether SLIT2 overexpression has a general cytotoxic rather than growth-suppressive effect, we analyzed the response of the unmethylated HCC38 breast tumor cell line (normal level of expression) to SLIT2 conditioned medium. SLIT2 had no significant effect on this line, thus supporting the growth-suppressive role of SLIT2 (Fig. 6B).

![Fig. 4. Detection of aberrant hypermethylation of the SLIT2 promoter region using the MSP assay. Shown are the results of the MSP assay of the putative SLIT2 promoter using primers specific for amplification of either methylated (M) or unmethylated (U) templates from cancer cell lines or matched normal (N) or tumor (T) tissues.](image-url)

![Table 2 LOH and retention at the SLIT2 locus in methylated (M) and unmethylated (U) breast, NSCLC, and SCLC cancers](table-url)

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>LOH</th>
<th>Retention</th>
<th>UI</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>M</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>NSCLC</td>
<td>M</td>
<td>7</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>1</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>SCLC</td>
<td>M</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>4</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

UI, uninformative cases.
DISCUSSION

Tumor-acquired promoter hypermethylation has been recently shown as a common inactivation mechanism for TSGs in human cancers (25). We and others have shown that the new TSG RASSF1A is inactivated almost exclusively by promoter region hypermethylation in lung, breast, and kidney cancers (19, 20, 26) and that mutations, apart from infrequent germ-line missense substitutions, are rare. In this study we report that SLIT2 is a target gene for methylation and inactivation in lung and breast cancers. The SLIT2 promoter region is methylated in 36–53% of primary lung and breast tumors. SLIT2 locus methylation was associated with loss of gene expression in lung and breast cancer cell lines, and this was reversed by treatment with a demethylating agent. Furthermore, SLIT2 expression was also reduced in methylated primary breast tumors. Methylation of one allele of SLIT2 is mostly associated with deletion of the other allele. This observation is in agreement with Knudson’s model of biallelic inactivation of TSGs (27). The detection of SLIT2 methylation in 8–14% of histologically normal matched tissues may indicate that SLIT2 methylation occurs very early in cancer progression. However, it is equally possible to suggest that this detectable methylation is caused by the presence of invading tumor cells in the neighboring normal tissue. Mutaional analysis of the 37 coding exons of SLIT2 has failed to reveal any inactivating changes. However, the two missense changes we found could have a detrimental effect on the protein function because they occur in highly conserved amino acids. Further analysis of these missense changes is warranted.

Ectopic expression of SLIT2 in several breast cancer cell lines suppressed growth and reduced colony formation abilities. Additionally, secreted SLIT2 in conditioned medium from SLIT2-transfected COS-7 cells reduced the cell number and growth of these breast cancer cell lines. The mechanism of this growth suppression is unknown. Slit2 protein is reported to be proteolytically cleaved into two fragments. The NH2-terminus fragment is longer and is primarily membrane-associated. The shorter, COOH-terminus fragment is primarily secreted (5). In the conditioned medium assay, the inhibitory effect is presumed to be mediated by the COOH-terminal fragment (Fig. 6A). ROBO1 is thought to be the putative receptor for SLIT2 (8), but there are at least three more ROBO receptors. ROBO2 is also located at 3p12 (approximately 5 Mb distal to ROBO1). Homology searches identify ROBO3 as the mouse Rig-1 homologue, located at 11q24. ROBO4 was recently cloned from 11q24 and is thought to be involved in angiogenesis (28).

The SLIT family is one of four conserved families of axonal guidance cues that have prominent developmental effects. The other three are the netrins, the semaphorins, and the ephrins (29). Evidence is growing for the involvement of these guidance cues and their receptors in carcinogenesis. SEMA3B, a member of the semaphorin family, suppresses growth of adenocarcinoma cancer cell lines (30). SEMA3B also induces apoptosis and causes growth suppression of lung cancer cell lines (31), possibly through the p53 pathway (32). SEMA3B expression is frequently lost in lung cancer, and this loss is caused by hypermethylation of its promoter region (31). The netrin-1 receptor, DCC (deleted in colorectal cancer), is frequently inactivated in colorectal cancer (33). DCC loss of expression is also associated with promoter region hypermethylation in primary gastric cancer (34). We have recently shown that the putative SLIT2 receptor, DUTT1/ROBO1, is epigenetically inactivated in a subset of kidney and breast cancers (21). Most mice with an absence of DUTT1/ROBO1 exon 2 die because of delayed lung maturation, whereas the surviving mice develop bronchial epithelial hyperplasia (11). The finding of SLIT2 inactivation further strengthens the concept that ROBO1 loss or methylation will promote tumorigenesis.

In conclusion, aberrant promoter methylation and associated transcriptional silencing are now recognized as a major mechanism of TSG inactivation. SLIT2 resembles other TSGs such as RASSF1A in that epigenetic inactivation appears to be much more frequent than mutational mechanisms. SLIT2 methylation appears to be a frequent event in two leading cancer types. SLIT2 inactivation provides an additional example of the significance of human developmental signaling pathways for cancer biologists and suggests that additional studies of SLIT2 in other tumor types are indicated. In addition, other ROBO and SLIT human homologues may also prove to be implicated in the pathogenesis of human cancers. Finally, we note that Slit2 proteins function as secreted chemorepellants (6), suggesting that SLIT2 inactivation in human cancers might be amenable to therapeutic intervention by reversal of epigenetic inactivation and by local ad-

Fig. 6. Growth-suppressive effect of secreted SLIT2. A, detection by Western blot of the different SLIT2 fragments in COS-7 cells or conditioned medium (CM). The antibody against human SLIT2 detects the full-length protein (Mr 190,000) in addition to the NH2-terminal fragment (Mr ~140,000), both of which are indicated by the arrowheads. The anti-MYC antibody detects the full-length protein in addition to the COOH terminus fragment (Mr 50,000–60,000) indicated by the arrowheads. Both fragments could be detected with this antibody from cellular extracts; however, the full-length fragment is undetectable in cleared conditioned medium. B, phase-contrast image of T-47D cells after treatment with conditioned media from vector- or SLIT2-transfected COS-7 cells. Values are the mean ± 2 SD of at least three separate experiments, each calculated from triplicate plates. In each case, the vector control was set at 100%. SLIT2 conditioned medium contains, on average, 10 nM SLIT2.
ministration of SLIT2 (or functional homologues of SLIT2). In addition to opportunities to develop novel therapeutic products, the detection of SLIT2 methylation in spurious or breast aspirates may provide novel diagnostic markers for lung and breast cancer.

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