**SLIT2 Axon Guidance Molecule Is Frequently Inactivated in Colorectal Cancer and Suppresses Growth of Colorectal Carcinoma Cells**

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**ABSTRACT**

We have shown recently that SLIT2 has tumor suppressor activity and that it is epigenetically silenced in >40% of lung and breast tumors. In this study, we have analyzed the methylation status of SLIT2 in primary colorectal cancers and matching normal colorectal mucosa. SLIT2 promoter region methylation was found in 23 (72%) of 32 primary colorectal cancers. In contrast, normal colorectal mucosa from the same patients exhibited significantly lower levels of SLIT2 promoter region hypermethylation. SLIT2 methylation was reversed and expression restored by treating colorectal tumor cell lines with the demethylating agent 5-aza-2'-deoxycytidine. Loss of heterozygosity at 4p15.1–15.3 region has been shown to occur early in colorectal carcinoma (10). Microsatellite markers from this region also show LOH in mesothelioma, small cell lung cancer, non-small cell lung cancer, and breast tumors (11, 12). We already have shown that SLIT2 is a good candidate for a TSG (tumor suppressor gene) in lung and breast cancer, two cancer types exhibiting LOH at 4p15. SLIT2 could also be implicated in CRC pathogenesis based on the frequency of 4p15 deletions in CRC. Therefore, we have analyzed a panel of colorectal tumor cell lines and primary tumors with matching histologically normal tissue for the presence of SLIT2 methylation. We used direct sequencing of a region in the putative SLIT2 promoter in an attempt to distinguish better between the levels of SLIT2 methylation in normal versus tumor DNA. We also screened the same panel for ROBO1 methylation and looked for a correlation between SLIT2 methylation and the inactivation of ROBO1/RASSF1A/p16/K-RAS. We also sought to determine whether SLIT2-induced growth suppression is mediated, at least in part, by the process of apoptosis.

**INTRODUCTION**

CRC is the second most common adult cancer in the Western world. CRC arises through several mechanisms (1). Microsatellite instability, caused by mismatch repair defect, is common in familial CRCs (hereditary nonpolyposis CRCs). Sporadic CRCs, on the other hand, have frequent alterations in APC/C-β-catenin pathway, frequent activating mutations in the K-RAS oncogene, and inactivating p53 mutations (1). CpG island hypermethylation is another mechanism of gene inactivation in CRC (1). Genes such as p16, THBS1, and hMLH are frequently found to be methylated in sporadic CRC. The term CIMP was recently described as a distinct pathway for colorectal cancers. In contrast, normal colorectal mucosa from the same patients exhibited significantly lower levels of SLIT2 promoter region methylation. SLIT2 methylation was reversed and expression restored by treating colorectal tumor cell lines with the demethylating agent 5-aza-2'-deoxycytidine. Loss of heterozygosity at 4p15.1–15.3 region has been shown to occur early in colorectal carcinoma (10). Microsatellite markers from this region also show LOH in mesothelioma, small cell lung cancer, non-small cell lung cancer, and breast tumors (11, 12). We already have shown that SLIT2 is a good candidate for a TSG (tumor suppressor gene) in lung and breast cancer, two cancer types exhibiting LOH at 4p15. SLIT2 could also be implicated in CRC pathogenesis based on the frequency of 4p15 deletions in CRC. Therefore, we have analyzed a panel of colorectal tumor cell lines and primary tumors with matching histologically normal tissue for the presence of SLIT2 methylation. We used direct sequencing of a region in the putative SLIT2 promoter in an attempt to distinguish better between the levels of SLIT2 methylation in normal versus tumor DNA. We also screened the same panel for ROBO1 methylation and looked for a correlation between SLIT2 methylation and the inactivation of ROBO1/RASSF1A/p16/K-RAS. We also sought to determine whether SLIT2-induced growth suppression is mediated, at least in part, by the process of apoptosis.

**MATERIALS AND METHODS**

**Patients and Samples.** A total of 32 CRC samples and their matching histologically normal mucosa plus 6 colorectal tumor cell lines were analyzed; these have been described previously (13). All of the relevant clinical information for primary tumors and tumor cell lines was available.

**Bisulfite Modification and Methylation Analysis.** Bisulfite DNA sequencing was performed as described previously (14). 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) in hydroquinone (Sigma) in a thermocycler (Hybaid) for 30 s at 99°C and for 15 min at 50°C for 20 cycles. 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 (http://www.genomatix.de). This region is from –761 to –212 relative to the translation start site. The region was amplified from cell lines or tumors using the primers SL2MOD4F (5′-GGGAGGTGGGATTGTATTTAGATTT-3′) and primer SL2MOD4R (5′-CAAATCCCTTCTAAAAACACTTTTATACCTTAAA-3′) as we have described previously (8). One-fifth volume of the PCR reaction (with primers SL2MOD4F and SL2MOD4R) was used in a nested PCR reaction with 30 cycles using primer SL2-SP-F (5′-AGTTTAGAGTYGTGGTATTTAGG-3′) and the primer SL2-SP-R (5′-CCCATCAAAATATCCCTTATT-3′), where Y is C+T and R is A+G. These primers amplify a region where we have found most of the methylated CpGs in the putative SLIT2 promoter to be concentrated. The PCR conditions for both the first and second PCR were 95°C for 10 min, followed by 30–40 cycles of 1 min denaturation at 95°C, 1 min
methylated CpGs; 1–14, the positions of the CpG dinucleotides analyzed. C, /H11002 partial methylation; SLIT2 sequence of the guidelines.

‘after treatment and then every 3 days. RNA was prepared 10 days after the primers 5'-GGTGTCCTCTGTGATGAAGAG-3' /H11032 /H11032 previously (15).

the maximum value of 2. A height along the entire sequence and subtracting the background G peak. The methylated CpG sequences after normalizing the A peak for the average peak levels using ABI BigDye cycle sequencing kit. The ratio of methylated alleles concentrated and purified using QIAquick PCR Purification columns (Qiagen).

° – C, and 2 min extension at 74 °C, and 2 min extension at 74 °C. The demethylating agent, 5-aza-dC (Sigma) was freshly maintained in DMEM growth medium (Invitrogen) supplemented with 10% FCS at °C.

24 h after treatment and then every 3 days. RNA was prepared 10 days after treatment using the RNeasy kit (Qiagen) according to the manufacturer’s guidelines. SLIT2 expression was detected by reverse transcription-PCR using the primers 5'-GGTGTCCTCTGTGATGAAGAG-3' and 5'-GTGTATTAGAGAGACACACCTG-3'. The expected product size was 387 bp. Expression of GAPDH was used as a control. The GAPDH primers were described previously (15).

Microsatellite Repeat Analysis. PCR amplification of the dinucleotides repeats microsatellite sequence of marker D4S1546 was performed on the methylated colorectal carcinomas. The D4S1546 marker was the nearest possible marker (within 100 kb) available to the SLIT2 gene on 4p15.2 according to Genome Databases 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 units 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.

Plasmid Constructs and Growth Suppression Analysis. The SLIT2 expression construct was made by cloning the full length of the human Slit2 protein) was prepared by transient transfection of COS-7 cells of SLIT2 staining with crystal violet. 10 nM SLIT2 -5-aza-dC conditioned medium (with ~10 nm SLIT2 protein) was prepared by transient transfection of COS-7 cells of SLIT2- or vector-only plasmids. Forty-eight h after transfection, the presence of SLIT2 was confirmed by Western blotting, and medium was cleared by centrifugation and added to 5 × 10^6 cells seeded 24 h earlier in each well of 6-well plates. After 4 days, the viable cells (as determined by trypan blue exclusion) in each indicated treatment were counted using a hemocytometer.

Detection of Apoptosis. SW48 cells undergoing early stages of SLIT2-induced apoptosis were detected by their increased ability to bind Annexin V-phycoerythrin conjugate (PharMingen). Cell necrosis was detected by the incorporation of 7-aminocoumarin D dye. Labeled cells were detected by fluorescence-activated cell sorting (FACS) analysis on a Coulter Epics flow cytometer. TUNEL activity was detected in SW48 cell line exposed to SLIT2-conditioned medium for 48 h using the In Situ Cell Death Detection kit (Roche) according to the manufacturer’s instruction. These experiments were done in replicates and repeated three times.

Statistical Analysis. Comparisons were made by Fisher’s exact test or t test when appropriate. P values of <0.05 were taken as statistically significant.

RESULTS

Methylation of SLIT2 Promoter Region Correlates with Lack of Expression. In previous work, we have shown that the SLIT2 gene was silenced by hypermethylation of its promoter region in a significant percentage of breast and lung cancers. To elucidate the role of SLIT2 in CRC pathogenesis, we analyzed the methylation pattern of 6 CRC cell lines (all of the cell lines used in this study are microsatellite instability [+]) and 32 CRC tumor/normal pairs. SLIT2 promoter region CpG island was found to be hypermethylated in five of these tumor cell lines, hypermethylation correlated with expression status, and all of the tumor cell lines with promoter methylation demonstrated reduced or absent mRNA expression, depending on the extent of methylation (Fig 1A, left panel). To confirm that methylation was respon-

![Diagram of methylation analysis of SLIT2](https://example.com/diagram.png)

Fig. 1. Methylation analysis of SLIT2. A, left, expression analysis of SLIT2 mRNA in CRC cell lines and its correlation with methylation status. +, complete methylation; +/-, partial methylation; –, no methylation. Right, reverse transcription-PCR analysis of SLIT2 expression before (−) or after (+) treatment with 10 μM 5-aza-dC for 10 days. The human glioma cell line U343, which is unmethylated for SLIT2, was used as a negative control for the 5-aza-dC effect on demethylation. Expression of GAPDH was used as a control. B, sequence of the SLIT2 promoter region showing the relative positions of the primers used for amplification. The primers sequences are given in “Materials and Methods.” Positions 1–14, the positions of the CpG dinucleotides analyzed. C, schematic representation of the methylation levels as detected in the indicated tumor cell lines. □, unmethylated CpGs; ■, methylated CpGs; partially filled squares, partially methylated CpGs.
sible for silencing SLIT2 gene expression, we treated three CRC cell lines with the demethylating agent 5-aza-dC. SLIT2 expression was restored in cell lines with absent or weak SLIT2 expression after 5-aza-dC treatment (Fig. 1A, right panel), whereas the unmethylated glioma cell line demonstrated no difference in SLIT2 expression before or after treatment.

In our previous work, we have found SLIT2 methylation to concentrate in a short region within the putative promoter (Fig. 1B). We designed bisulfite-modified DNA-specific primers to amplify this short region and to use it in direct sequencing analysis. Using such a short region (146 bp) minimizes the problems associated with sequencing directly from bisulfite-modified DNA PCR products using the ABI BigDye chemistry. One major problem is the background G levels (obtained from the reverse sequence) caused by the ABI software compensating for the lower-than-expected G ratio. We have validated all of the sequencing results by a combination of COBRA (Combined Bisulfite Restriction Analysis) and sequencing of individual clones from PCR products cloned into pGEM T-easy vector. In our experience, we have found that direct sequencing allows more accurate estimation of the level of methylation of SLIT2 in comparison with using methylation-sensitive PCR or COBRA. The results of the sequencing analysis of the CRC cell lines are shown in Fig. 1C. The CRC cell lines SW48, DLD1, and LoVo are heavily methylated for this region, whereas the CRC line LS174T shows partial methylation, and the glioma cell line U343 shows no methylation (Fig. 1C). These results correlate very well with our expression analysis (Fig. 1A).

We have applied the same strategy to show the relative methylation levels between tumor tissues and neighboring histologically normal mucosa (Fig. 2). We found tumor-specific methylation of SLIT2 promoter region in at least 23 (72%) of 32 CRC tumors. We also found a weak level of SLIT2 methylation in almost all of the corresponding normal mucosa samples. No significant methylation could be found in the normal samples that had no methylation in the corresponding tumor. In our previous study, we found that there was no detectable SLIT2 methylation in lymphocytes from healthy individuals (4). The presence of SLIT2 methylation in tumor cases did not correlate with tumor grade or patient’s age. LOH analysis at D4S1546 (which maps within 100 kb of SLIT2) demonstrated allele loss in 39% of informative CRC cancers with SLIT2 methylation. We have previously shown that SLIT2 is mainly silenced by hypermethylation and that we did not find any somatic mutations in the coding region of SLIT2 in a panel of lung and breast cancers, and, therefore, it is unlikely that SLIT2 is inactivated by mutations in a significant manner in CRC.

We also analyzed this CRC panel for ROBO1/DUTT1 methylation and found only 6 (19%) of 32 tumors to have a methylated ROBO1/DUTT1 promoter region (Table 1; Fig. 3). This observation was similar to ROBO1/DUTT1 methylation levels that we have found previously in breast and kidney tumors (15). There was no significant correlation between SLIT2 methylation and ROBO1/DUTT1 methylation in the tumors analyzed. There was also no significant correlation with inactivation of the ROBO1/DUTT1 gene and tumor grade or patient’s age.

We had information on RASSF1A and p16 methylation status and
K-RAS mutation status in this CRC panel from a previous study (13). We found no significant correlation between SLIT2 methylation and RASSF1A/p16K-RAS inactivation (Table 1; Fig. 3).

**SLIT2 Induces Growth Suppression and Apoptosis.** Having demonstrated that epigenetic inactivation of SLIT2 is frequent in CRC, we investigated tumor suppressor activity of SLIT2 in *in vitro* colony formation assays. A wild-type SLIT2 expression plasmid or the pSecTagB empty vector were transfected into SW48 and LoVo tumor cell lines. Both constructs expressed a zeocin resistance gene. Transfection with SLIT2 significantly reduced (*P* < 0.0055) the number of zeocin-resistant colonies by >70% compared with transfection with empty vector in both of the cell lines tested (Fig. 4A). This effect was consistent through three independent experiments and using three independent plasmid DNA preparations. As additional evidence for the ability of SLIT2 to suppress growth, conditioned medium from COS-7 cells transfected with SLIT2 causes a significant growth inhibition (*P* < 0.0001) in relation to control. SLIT2-conditioned medium causes up to 65% growth inhibition in the SW48 cell line (Fig. 4B). In addition, SLIT2 induces apoptosis as detected by the increase in Annexin V-PE labeling in SW48 (up to 20%) after treatment with SLIT2-conditioned medium for 48 h (Fig. 5A). SLIT2-conditioned medium caused an average increase of 11.50 ± 1.79% of annexin V-positive/7-AAD negative cells. This effect was significant and consistent throughout three experiments (*P* < 0.008). SLIT2-induced apoptosis could also be detected using TUNEL. The average increase in TUNEL positive cells after exposure to SLIT2 was 7.7 ± 0.65% compared with control (*P* < 0.0001; Fig. 5B).

**DISCUSSION**

In our previous work (4, 14), we have analyzed the role of the *SLIT2/ROBO1* pathway in human cancers. We initially looked for a possible role of *ROBO1* in tumor suppression because it was cloned from the 3p12 region. The 3p12 region exhibits high levels of LOH and overlapping homozygous deletions in human cancers. However, *ROBO1* was not inactivated by somatic mutations but was epigenetically silenced in ~19% of breast and kidney cancers and in a small number of lung cancers. Analysis of the *SLIT2* genetic and epigenetic status revealed a higher level of inactivation (>40%), mainly by hypermethylation, in lung and breast cancers, and we correlated this methylation with reduced expression in tumor cell lines and primary tumors. Therefore, we hypothesized that haploinsufficiency of *DUFT1* coupled with haploinsufficiency/inactivation of its putative ligand SLIT2 could be promoting tumor progression and metastasis.

In this study, we provide evidence for SLIT2 inactivation by promoter region hypermethylation in the majority of CRC samples analyzed. Our data support the revised Knudsen two-hit theory (17) with one allele being inactivated by methylation and the other by LOH. Methylation of *SLIT2* found in the neighboring normal mucosa, in which the tumor is methylated to a significantly higher degree, may indicate that *SLIT2* methylation is an early event in CRC tumorigenesis. However, in view of the role of *SLIT2* in controlling axon and leukocytes migration,
it is possible that methylated SLIT2 from migrating tumor cells is being detected. All of our samples come from patients ages 47–96 years old with an average age of 74 and a median age of 76 years old. Therefore, all of the samples were collected from “older” patients. Although there is no statistical correlation between age and SLIT2 methylation, there were no samples from young patients in our study; therefore, we cannot exclude the possibility that the methylation of SLIT2 in normal mucosa is associated with aging, as has been described for the methylation of the estrogen receptor in CRC (18). We also demonstrate ROBO1 methylation in CRCs at a frequency equal to that with breast and kidney tumors (19%). SLIT2 methylation did not correlate with the methylation of ROBO1 promoter region 1p6/H110051a or with K-RAS mutations.

The SLIT family is one of four conserved families of axonal guidance cues that have prominent developmental effects. The fact that these molecules are secreted provide a novel therapeutic potential. The other three are the netrins, the semaphorins, and the ephrins (19). 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 (20). SEMA3B also induces apoptosis and causes growth suppression of lung cancer cell lines (21), possibly through the p53 pathway (22). SEMA3B expression is frequently lost in lung cancer, and this loss is caused by hypermethylation of its promoter region (21). The Netrin-1 receptor, DCC, is frequently inactivated in CRC (23). DCC loss of expression is also associated with promoter region hypermethylation in primary gastric cancer (24).

Ectopic expression of SLIT2 in CRC cell lines suppressed growth and reduced colony formation abilities. Additionally, secreted SLIT2, which consists predominantly of the smaller COOH-terminal fragment, in conditioned medium from SLIT2-transfected COS-7 cells, reduced cell number and growth of these CRC lines. We have shown previously that this effect could be seen in breast tumor cell lines and that it is not attributable to general SLIT2-induced cytotoxicity (4). We have studied SLIT2-induced apoptosis in an attempt to investigate the mechanism of SLIT2-induced growth suppression. We show that SLIT2 induces apoptosis and cell death as indicated by the increased Annexin V binding and TUNEL staining. The mechanism by which SLIT2 induces apoptosis is unclear. If this apoptosis is induced through the DCC pathway, then it is possible to consider caspase 3 and caspase 9 as likely candidates for mediating SLIT2-induced apoptosis (8).

In conclusion, aberrant promoter methylation and associated transcriptional silencing is now recognized as a major mechanism of TSG inactivation. SLIT2 methylation appears to be a very frequent and possibly early event in CRC and is independent from p16/RASSFLA/ROBO1 methylation and K-RAS mutation status. The demonstration that SLIT2 can actively induce apoptosis suggests that this gene may have an important role in controlling the growth and migration of colorectal cells. SLIT2 provides an excellent candidate for a 4p15.2 TSG for sporadic CRC.

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