Epigenetic inactivation of tumour suppressor genes (TSGs) by promoter region CpG island hypermethylation is now well documented and several TSGs have been demonstrated to be inactivated by this mechanism (reviewed in Jones and Baylin, 2002; Dammann et al., 2001; Burbee et al., 2002). These proteins are widely expressed in mammalian tissues and the expression is not confined to neurons. Hence, they may have other yet unidentified roles. Slits are secreted proteins that are ligands for the Robo receptors (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). Recently, Slit was shown to inhibit leukocyte chemotaxis, this inhibition appears to be mediated by Robo (Wu et al., 2001). In mammals, four Robo genes have so far been identified, Robo1, Robo2, Rig-1 (Robo3) and magic Robo (Robo4) (Kidd et al., 1998; Sundaresan et al., 1998; Yuan et al., 1999; Huminiecki et al., 2002). In humans, ROBO1 is located at 3p12 within a critical region of overlapping homozygous deletions in lung and breast cancers (Sundaresan et al., 1998). This region also demonstrated a high frequency of allele loss in lung, kidney and breast cancers. Majority of mice with deletion of exon 2 of Robo1 die at birth because of delayed lung maturation, the surviving mice develop bronchial hyperplasia (Xian et al., 2001). In an earlier study, we demonstrated that there were no inactivating somatic mutations in ROBO1 in lung and breast cancers, but a CpG island in the 5′ region of ROBO1 was hypermethylated in breast and kidney tumours (Dallol et al., 2002b). We went on to analyse
the ligand SLIT2 located at 4p15.2 for genetic/epigenetic inactivation in tumours. The 4p15.2 region shows frequent allele loss in lung, breast, colorectal and head and neck cancers. SLIT2 promoter region CpG island was found to be frequently hypermethylated in lung, breast, colorectal and glioma tumours, while somatic mutations were not found (Dallol et al., 2002a, 2003a, b). Furthermore, we demonstrated in vitro growth suppression when SLIT2 was expressed in tumour cell lines that had no endogenous SLIT2 expression due to methylation. A recent paper demonstrated that in mice slit2 homozygous deficiency was lethal (Plump et al., 2002). In this report, we analysed the methylation status of 5’ CpG islands for the remaining SLIT gene family members (SLIT3 and SLIT1) in human cancers.

MATERIALS AND METHODS

Patients and samples

A total of 60 glioma samples, plus seven glioma tumour cell lines (T17, U87-MG, A172, U343, HS683, U373, H4) were analysed for methylation. Among the glioma samples, 40 were classified as glioblastoma multiforme. The remaining gliomas were collected randomly and consisted of all grades. In addition, 32 colorectal cancer samples and their matching histologically normal mucosa, six colorectal tumour cell lines (SW48, HCT116, LS411, LS174T, DLD1, LoVo), 15 lung tumour cell lines, 32 invasive ductal breast carcinoma plus 29 breast tumour cell lines were also analysed for methylation. These have been described previously (Dallol et al., 2002a, 2003a, b).

Bisulphite modification and methylation analysis

Bisulphite DNA sequencing was performed as described previously (Agathanggelou et al., 2001). Briefly, 0.5–1.0 μg of genomic DNA was denatured in 0.3 M NaOH for 15 min at 37°C. Unmethylated cytosine residues were then sulphonated by incubation in 3.12 M sodium bisulphite (pH 5.0) (Sigma, Dorset, UK) and 5 mM hydroquinone (Sigma) in a thermocycler (Hybaid) for 15 s at 99°C and 15 min at 50°C for 20 cycles. Sulphonated DNA was then recovered using the Wizard DNA cleanup system (Promega, Southampton, UK) according to the manufacturers’ instructions. The DNA was desulphonated by addition of 0.3 M NaOH for 10 min at room temperature. The converted DNA was then ethanol precipitated and resuspended in water.

Table 1 Expression and methylation primer sequences and PCR conditions

| Primer name | Primer sequence (5’ to 3’) | Annealing temp. for PCR (°C) | MgCl₂ conc. (mM) | Predicted product size (bp) |
|-------------|---------------------------|-----------------------------|-----------------|---------------------------|
| SLIT3 COBRA F | GGT-TAG-TTT-ATT-YGG-TYG-TTT-YGT-G TT-TTA-GT | 55 | 1.5 | 394 |
| SLIT3 COBRA R | TAC-CCA-CCC-RAA-AAC-CAT-AAT-ATA-CAA-AA | | | |
| SLIT3 COBRA RN | CCA-CTC-CTA-AAA-AACT-ACC-TCT-A | | | |
| SLIT1 COBRA F | GGT-TAT-TTT-TTT-TAT-YGG-TTG-TTG-GGA-GT | 57 | 1.5 | 370 |
| SLIT1 COBRA R | TAA-CAT-ACC-RTA-ATT-CRR-ATA-CAAATA-CAA-AA | | | |
| SLIT1 COBRA RN | GGY-AAGAT-GTA-GAG-TYGT-ATT-GTT-TT | | | |
| GAPDH expression F | TGA-AGT-TGC-CGA-CCA-AGG-GAG-TTG-GGT-TTG-GT | 60 | 1.5 | 982 |
| GAPDH expression R | CAT-GTG-CGC-CAT-GAG-GTC-CAC-CAC | | | |
| SLIT3 expression F | CAA-CTG-TGC-CGA-GGG-GTA-CTG-GG | 65 | 1.5 | 419 |
| SLIT3 expression R | ACG-GG-CCA-TTA-GGA-ACA-CGC-GAG-GG | | | |
| SLIT1 expression F | GTG-ACT-GCA-CTG-ACT-GTA-AGA-AC | 55 | 1.5 | 422 |
| SLIT1 expression R | GTA-CAG-CTC-AAC-TGC-AAT-GT | | | |

Figure 1. Quantitative real-time RT-PCR was used to measure SLIT1 and 3 expression levels in 20 normal human tissues. See Materials and Methods for real-time RT-PCR details. RPLPO gene transcript was used as the endogenous RNA control and each sample was normalised on the basis of its RPLPO content.
Combined bisulphite restriction analysis (COBRA) and sequencing

All reactions were performed on a thermocycler (Hybaid) and with HotStar Taq Polymerase (Quiagen, West Sussex, UK). The promoter methylation status of SLIT3 and SLIT1 were determined using the COBRA method followed by sequencing to confirm methylation and ascertain the extent of methylation. The cycling conditions were as follows: initial denaturation for 10 min at 95°C, followed by 25–35 cycles of 1 min at 95°C, 1 min at annealing temperature and 2 min at 74°C with a final extension for 5 min at 74°C using the forward and reverse primers. The reaction volume of 20 μl contained 40 ng bisulphite-modified DNA, 1× PCR Buffer containing 1.5 mM MgCl₂ (Qiagen), 0.2 mM dNTPs, 0.4 μM each primer and 0.5 U HotStar Taq (Qiagen). Then, 1 μl of this reaction was used in a seminested PCR reaction (50 μl) using forward and reverse nested primers in the case of SLIT3 and reverse and forward nested primers for SLIT1. The same PCR programme and concentration of reagents were used as before. The annealing temperature, MgCl₂ concentration and sequences for the gene primers are listed in Table 1 (Y = C or T and R = A or G). All PCR products were assayed for methylation by incubation with BstUI at 60°C or TaqI at 75°C for 2 h before visualisation on a 2% agarose gel with added ethidium bromide. The CpG island methylation status for SLIT3 was determined by cloning PCR products into pGEM T-Easy vector (Promega – according to manufacturers’ instructions). At least five clones from each PCR product were then prepared for sequencing. SLIT3 Colony PCR products were purified using the QIAquick PCR Purification Columns (Qiagen – according to manufacturers’ instructions) and then reamplified using ABI BigDye Cycle Sequencing Kit (Perkin-Elmer, Warrington, UK) with the reverse nested primer (as shown in Figure 2A).

The SLIT1 COBRA PCR products were purified and then
Figure 3 (A) SLIT3 CpG island COBRA PCR products were cloned and sequenced from breast (MCF7, HCC712, HCC38 and HCC70) and colorectal (SW48) tumour cell lines. For each tumour cell line, several clones were sequenced and the methylation status for the first 30 CpGs is shown. White and black squares represent unmethylated and methylated CpGs, respectively. Partially filled squares represent partially methylated CpGs. (B) Expression of SLIT3 in methylated breast (MCF7) and colorectal (SW48) cell lines before and after treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-aza dC). Gene expression was restored by 5-aza-dC (+) in methylated cell lines that lacked SLIT3 expression. GAPDH and the unmethylated glioma cell line T17 were used as positive controls to ensure RNA integrity and equal loading.

Cell lines and 5-aza-2′ deoxycytidine treatment

Breast, colorectal and glioma tumour cell lines were routinely maintained in RPMI 1640 (Invitrogen, San Diego, CA, USA) supplemented with 10% FCS at 37 °C, 5% CO2. 5–10 × 10^5 cells were plated and allowed 24 h growth before addition of 5-aza-2′-deoxycytidine (Sigma). The medium was changed 24 h after treatment and then every 3 days. RNA was prepared at 5 and 7 days after treatment using the Rnasy kit (Qiagen) according to manufacturers’ instructions.

Expression analysis

Breast, colorectal and glioma cell line were treated with 5 μM demethylating agent 5-aza-2′-deoxycytidine freshly prepared in ddH2O and filter-sterilised. Extracted RNA (1 μg) was used as a template for cDNA synthesis using SuperScript™ III RNase H− Reverse Transcriptase (Invitrogen – according to the manufacturers’ instructions). In total, 2 μl (10%) of the first strand reaction was used for PCR (Invitrogen – according to the manufacturers’ instructions). Primers used for SLIT1, SLIT3 and GAPDH RT – PCR are described in Table 1. The PCR thermocycle (Hybaid) consisted of an initial denaturation of 10 min at 95 °C followed by 35 cycles of 95 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s and a final extension of 5 min at 72 °C. PCR products were visualised on a 2% agarose gel with added ethidium bromide.

RESULTS

SLIT1 and SLIT3 expression analysis in normal tissues using quantitative real-time RT–PCR

We investigated the expression pattern of SLIT1 and SLIT3 genes in a wide range of normal human tissues using quantitative real-time RT–PCR (see Materials and Methods). SLIT3 was expressed in majority of tissues analysed, with the highest expression in skin, brain cerebellum and lung and lowest expression in fetal liver,
bone marrow and stomach. While, SLIT1 expression was much more restricted (brain and nervous system) (Figure 1).

Epigenetic inactivation of the SLIT3 gene in tumour cell lines

The SLIT3 putative promoter region was predicted by Promoter Inspector software (http://www.genomatrix.de). This region is from −576 to + 9 relative to the translation start site. This region fulfilled the criteria of a CpG island with a GC content of 77% and an observed:expected CpG ratio of 0.86 (CpG plot programme at http://www.ebi.ac.uk/Tools/). We investigated the methylation status of this 5′ CpG island associated with the SLIT3 gene in various human tumour cell lines. For this analysis, we utilised the COBRA assay on bisulphite-modified DNA. Figure 2A shows the sequence of the region analysed and the primers used (sequence shown is reverse strand bisulphite modified and all CG methylated), TaqI and BstUI sites are underlined. This CpG island was found to be hypermethylated in 12 out of 29 (41%) breast, one out of three (33%) NSCLC, zero out of 12 (0%) SCLC, two out of six (33.3) colorectal and in two out of seven (29%) glioma tumour cell lines. The COBRA PCR products were cloned and several clones of each tumour cell line were sequenced to determine the pattern and extent of methylation. As seen in Figure 3A, majority of the 30 CpG dinucleotides were hypermethylated for MCF-7, HCC712 and HCC38 (breast tumour lines) and SW48 (colorectal tumour line), while breast tumour cell line HCC70 was unmethylated. SLIT3 expression was restored in tumour lines that were heavily methylated (MCF-7 and SW48) by treating the cell lines with 5aza-2-deocytidine (Figure 3B), while the unmethylated glioma tumour cell line T17 did not show any change in expression.
Epigenetic inactivation of the SLIT3 gene in primary tumours

We then analysed the methylation status of the above CpG island in primary tumours using COBRA analysis followed by sequencing of the cloned PCR products. Five out of 32 (16%) breast tumours were found to be methylated for SLIT3. Using real-time RT–PCR, we demonstrated that the SLIT3 methylation in breast tumours correlated with reduced SLIT3 expression as compared to unmethylated breast tumours (Figure 4A).

SLIT3 CpG island was also hypermethylated in 12 out of 32 (37.5%) colorectal tumours and in 21 out of 60 (35%) glioma primary tumours. Since the tumour samples used in this study were not microdissected, in majority of the primary tumors unmethylated bands were also detected. No methylation was found in corresponding normal tissues from the colorectal or glioma patients or in the DNA isolated from normal brains. Similar to the tumour cell line data, sequencing of cloned PCR products confirmed that majority of the 30 CG dinucleotides analysed were methylated in primary tumours (Figure 4B, C).

Epigenetic inactivation of the SLIT1 gene in gliomas

The SLIT1 CpG island was predicted to be from −574 to +192 and had a GC content of 71% and had an observed : expected CpG ratio of 0.81 (CpG plot programme at http://www.ebi.ac.uk/Tools/). This region overlapped with the SLIT1 putative promoter region predicted by Promoter Inspector software (http://www.genomatix.de). Since SLIT1 expression is neuronal specific, we analysed the methylation status of the 5′ CpG island of the SLIT1 gene in glioma tumour cell lines and primary tumours by COBRA and direct sequencing of bisulphite-modified DNA (Figure 2B). Five out of six (83%) glioma tumour lines were methylated, while only two of 20 (10%) glioma tumours demonstrated SLIT1 methylation (Figure 5A, B). No methylation was found in DNA isolated from normal brains. SLIT1 expression was restored/upregulated in five glioma tumour lines (methylated for SLIT1) by treatment with 5-aza-2′-deoxycytidine (Figure 5C).

DISCUSSION

The Slit genes encode ligands for the roundabout (robo) receptors. The Slit–Robo interactions mediate the repulsive cues on axons and growth cones during neural development. The Slit family comprises of large extracellular matrix-secreted and membrane-associated glycoproteins with multiple functional domains (reviewed in Brose and Tessier-Lavigne, 2000). Slit genes have been identified in Drosophila, Caenorhabditis elegans, Xenopus, chickens, mice, rats and humans. There are three known mammalian.....
SLIT genes (SLIT1, SLIT2, SLIT3) located on chromosome 10q23.3–q24, 4p15.2 and 5q35–q34, respectively. slit2 and slit3 genes are expressed in neuronal as well as nonneuronal tissues, while slit1 expression is specific to the brain (Wu et al, 2001 and this report). Slit2 homozygous deficiency in mice is lethal, while Slit1 and Slit3 homozygous mice are viable (Plump et al, 2002; Yuan et al, 2003).

In our earlier studies, we demonstrated that the ligand (SLIT2) for robo1 receptor was frequently methylated in lung, breast, colorectal and glioma tumours and that the methylation correlated with loss of SLIT2 expression. More recently, we demonstrated SLIT2 methylation in neuroblastoma, Wilms' tumour and renal cell carcinoma (Astuti et al, 2004). Furthermore, in in vitro assays, SLIT2 suppressed tumour growth (Dallol et al, 2002a, 2003a,b). SLIT3 is located at 5q35-3q4, which is a frequent region of allelic loss in colorectal and lung cancers (Girard et al, 2000; Goel et al, 2003). We have now demonstrated that SLIT3 5' CpG island similar to SLIT2 is frequently hypermethylated in colorectal and glioma tumours and less so in breast tumours. And loss of SLIT3 expression can be reversed by treatment with a demethylating agent. While SLIT1 gene is frequently methylated in glioma tumour lines but at low frequencies in glioma tumours, hence SLIT1 may play a role in late gliomagenesis.

Slits, netrins, semaphorins and the ephrins constitute conserved families of axonal guidance cues that have prominent developmental effects. Recently, SEMA3B was also demonstrated to be inactivated in lung cancer by promoter region hypermethylation (Tomizawa et al, 2001; Kuroki et al, 2003). Re-expression of SEMA3B inhibited lung cancer cell growth and induced apoptosis.

The finding of epigenetic inactivation in various human cancers of ROBO1, SEMA3B, SLIT2 and now SLIT3 and to a lesser extent SLIT1, all of which are involved in axon and cell migration in Drosophila and vertebrates, suggests a novel, and common underlying theme for these molecules in tumour suppression.

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