SLIT2 promoter methylation analysis in neuroblastoma, Wilms’ tumour and renal cell carcinoma

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The 3p12.3 RASSF1A tumour suppressor gene (TSG) provides a paradigm for TSGs inactivated by promoter methylation rather than somatic mutations. Recently, we identified frequent promoter methylation without somatic mutations of SLIT2 in lung and breast cancers, suggesting similarities between SLIT2 and RASSF1A TSGs. Epigenetic inactivation of RASSF1A was first described in lung and breast cancers and subsequently in a wide range of human cancers including neuroblastoma, Wilms’ tumour and renal cell carcinoma (RCC). These findings prompted us to investigate SLIT2 methylation in these three human cancers. We analysed 49 neuroblastomas (NBs), 37 Wilms’ tumours and 48 RCC, and detected SLIT2 promoter methylation in 29% of NB, 38% of Wilms’ tumours and 25% of RCC. Previously, we had demonstrated frequent RASSF1A methylation in the same tumour series and frequent CASP8 methylation in the NB and Wilms’ tumour samples. However, there was no significant association between SLIT2 promoter methylation and RASSF1A or CASP8 methylation in NB and RCC. In Wilms’ tumour, there was a trend for a negative association between RASSF1A and SLIT2 methylation, although this did not reach statistical significance. No associations were detected between SLIT2 promoter methylation and specific clinicopathological features in the tumours analysed. These findings implicate SLIT2 promoter methylation in the pathogenesis of both paediatric and adult cancers and suggest that further investigations of SLIT2 in other tumour types should be pursued. However, epigenetic inactivation of SLIT2 is less frequent than RASSF1A in the tumour types analysed.

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Methylation of CpG dinucleotides in the promoter regions of tumour suppressor genes (TSGs) produces transcriptional silencing is a major mechanism of TSG inactivation in many human cancers (Baylin et al, 1998; Jones, 1999; Tycko, 2000; Costello and Plass, 2001). The frequency of TSG inactivation by de novo methylation varies between TSGs and between cancers. Initially, epigenetic silencing of TSGs was reported as an alternative, but a minor mechanism of inactivation for TSGs such as RB1 and VHL (Gregor et al, 1994; Herman et al, 1994). However, recently a new class of TSGs has been recognised for which epigenetic silencing is the overwhelming mechanism of inactivation and somatic mutations are rare. This paradigm is exemplified by the RASSF1A 3p12.3 TSG, which is methylated in a wide range of human cancers (Dammann et al, 2000; Agathanggelou et al, 2001; Astuti et al, 2001; Burbee et al, 2001; Dreijerink et al, 2001; Morrissey et al, 2001).

Recently, we identified frequent promoter region hypermethylation of SLIT2 in lung and breast cancers (Dallol et al, 2002a). SLIT2 maps to 4p15.2 and encodes a human orthologue of the Drosophila Slit protein, a secreted glycoprotein, which regulates axon guidance, branching and neural migration during development of the central nervous system (Brose et al, 1999). Slit exerts its effects as a diffusible chemorepellent via its receptor Roundabout (Robo) (Stein and Tessier-Lavigne, 2001). In mammals, three distinct slit genes (slit1, slit2, and slit3) and three distinct robo genes (robo1, robo2, rig-1) have been identified. In lung and breast cancers, SLIT2 mutations were not detected despite frequent promoter methylation (similar to RASSF1A) (Dallol et al, 2002a). Methylation of RASSF1A was first reported in lung and breast cancers, but subsequently we and others demonstrated RASSF1A methylation in renal cell carcinoma (RCC) and in common childhood tumours such as neuroblastoma (NB) and Wilms’ tumour (Astuti et al, 2001; Dreijerink et al, 2001; Lo et al, 2001; Morrissey et al, 2001; Wagner et al, 2002). These findings prompted us to extend our studies of SLIT2 inactivation by studying SLIT2 promoter methylation in NB, Wilms’ tumour and RCC.
MATERIALS AND METHODS

Patients and samples

A total of 134 tumour samples were analysed (49 NBs, 37 primary Wilms’ tumours, and 48 adult RCCs). Details of the tumours have been published previously (Astuti et al., 2001; Wagner et al., 2002). DNA was extracted from tumour and normal tissues (blood or matched kidney) by standard methods.

Sodium bisulphite modification

Sodium bisulphite modification was carried out using an adapted method (Hereman et al., 1996). Genomic DNA (0.5–1 μg) was denatured at 37°C for 10 min in 0.3 M NaOAc. Unmethylated cytosines were sulphonated by incubation in 3.12 M sodium bisulphite/1 M hydroquinone (pH 5) at (95°C (30 s) 50°C (15 min)) × 20 cycles. The resulting sulphonated DNA was purified using the Wizard DNA clean-up system (Promega, Southampton, UK), according to the manufacturer’s instructions, except that DNA was eluted with distilled water (50 μl) at room temperature. Following elution, DNA was desulphonated in 0.3 M NaOAc for 5 min at room temperature, then the DNA was precipitated with NaOAc (5 μl of 3 M) and ethanol (125 μl of 100%) overnight at −20°C and resuspended in 50 μl distilled water.

Methylation-specific polymerase chain reaction (PCR)

The CpG island within the putative SLIT2 promoter has been described in detail previously (Dallol et al., 2002a). Methylation Specific PCR (MSP) analysis was performed essentially using primers and conditions as described in Dallol et al., 2002a). Combined Bisulphite Restriction Analysis (CoBRA) analysis was carried out as follows. In brief, the putative promoter region from nt −761 to −212 (relative to the translation start site) was amplified with primers reported previously (Slit2MODF (5′-GAGAGGTGGAGATATTAGATATTT-3′) and Slit2MODR2 (5′-GAAAATCTCCTTAAACACCTTATCTAAA-3′)). From this reaction, one out of 50 of the volume was used as a template for a nested PCR with primers Slit2MODF as above and Slit2MODR (5′-ACTAAACTTTACAACACTACTAAATAAATGAA-3′) to produce a 418 bp product (PCR conditions: 95°C for 10 min, followed by 30–40 cycles of 1 min denaturation at 95°C, 1 min annealing at 54°C, and 2 min extension at 74°C). PCR products were then digested by BstUI restriction enzyme to assess the methylation status of samples.

Sequencing of PCR products

MSP and CoBRA products were excised from agarose gels and extracted using the QIAquick Gel Extraction Kit (QIAGEN, Crawley, UK, West Sussex), according to the manufacturer’s instructions. Products were confirmed by direct sequencing from the forward PCR primer using ABI Prism® BigDye™ Terminators Cycle Sequencing Kit according to the manufacturer’s instructions and run using ABI 377 automatic sequencers.

5-aza-2’-deoxycytidine treatment of cell lines

5-aza-2’-deoxycytidine (5-aza-dC, Sigma, Poole, UK) was freshly prepared in ddH2O (at 2 mg ml−1) and filter-sterilised. A total of 0.5–1×106 cells were plated in 75 cm2 flask in RPMI 1640 media supplemented with 10% foetal calf serum and left to settle for 24 h (day 0). Kidney and NB cell lines were treated with 2 μM 5-aza-dC for a total of 5 days. Kidney and NB have a different rate of growth. Kidney tumour cell lines were treated with 5-aza-dC on days 1, 3 and 5 and harvested on day 6, with a medium change in days 2 and 4 (Nojima et al., 2001). Neuroblastoma cell lines were treated on days 1 and 4. The medium was changed 24 h after treatment and then every 3 days. RNA was prepared after treatment using the RNeasy kit (Qiagen) according to the manufacturer’s guidelines. SLIT2 gene expression was assayed by RT – PCR using the primers 5′-GGTGTCTCCTGTGATAGGACG-3′ and 5′-GTGTATTAG GAGACACACCTCG-3′, resulting in a product size 387 bp. As a control, the GAPDH primers used were: 5′-GACCCCTCAT GACCTCAACTACA-3′ and 5′-CTAAGCAGTTGTTGCGAGGA-3′, resulting in a PCR product of 369 bp.

Microsatellite repeat analysis – loss of heterozygosity

By searching GDB and the UCSC assembly of the human genome sequence, we identified D4S1546 as the closest marker to SLIT2 (within 100 kb). A 4p15.2 allele loss was assessed with the D4S1546 marker. (PCR conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 52°C (55°C for 30 s, and 72°C for 30 s and a final extension of 10 min at 72°C).

Statistical analysis

Fisher’s exact test was used as appropriate. P-values of <0.05 were considered to be statistically significant.

RESULTS

SLIT2 methylation status in NB

SLIT2 methylation status in NB was analysed in 49 primary NB tumours and 29% (14 out of 49) demonstrated SLIT2 CpG island promoter methylation (Figure 1A). Promoter CpG island methylation was confirmed by direct sequencing of five clones from one tumour (Figure 4B). We also analysed eight NB cell lines for SLIT2 methylation by restriction digestion and two cell lines (SK-N-F1 and SK-N-SH) were found to be partially methylated. SLIT2 methylation was detected in one out of 49 corresponding normal blood samples.

To investigate the 4p15.2 allelic status of NBs with SLIT2 methylation, we typed 13 methylated tumours for loss of heterozygosity (LOH) at D4S1546 that maps close to SLIT2. In all, 33% (four out of 11) of informative tumours demonstrated allele loss consistent with homozygous SLIT2 inactivation (Figure 2).

SLIT2 promoter methylation is associated with transcriptional silencing

To determine the relationship of SLIT2 promoter region CpG island methylation and SLIT2 transcript expression in the NB cell lines SK-N-F1 and SK-N-SH, we treated the cells with the demethylating agent, 5-aza-dC, for 5 days. The 5-aza-dC treatment significantly increased SLIT2 expression in both cell lines, but there was little or no change in the expression of GAPDH expression after the 5-aza-dC treatment (Figure 3).

SLIT2, CASP8 and RASSF1A methylation status in NB

Previously, we reported that RASSF1A and caspase 8 (CASP8) promoter methylation occurred in 55 and 40% of NBs, respectively. To determine whether there was any relationship between SLIT2 promoter methylation and de novo methylation of RASSF1A and CASP8 promoters, we compared the frequencies of RASSF1A and CASP8 methylation in tumours with and without SLIT2 methylation (using previously published RASSF1A and CASP8 methylation data (Astuti et al., 2001). CASP8 methylation was detected in 36% of SLIT2 methylated and 41% SLIT2 unmethylated tumours (P = 1.0). RASSF1A promoter methylation was more frequent in tumours with SLIT2 promoter methylation (77 vs 59%), but this did not reach statistical significance (P = 0.32).

Neuroblastoma clinicopathological features and SLIT2 methylation status

We compared the results of SLIT2 methylation status in our tumour series to the previously reported results for allelic loss
of 1p or 3p loss, N-myc amplification and 17q gain (Martinsson et al, 1997). There was no correlation between SLIT2 methylation and 1p allele loss (22% of SLIT2 methylated and 22% of unmethylated tumours, $P = 1.0$), 3p allele loss (25% vs 12%, $P = 0.57$), 17q gain (50% vs 65%, $P = 0.62$) or N-myc amplification status (15% vs 13%, $P = 1.0$). There was no association between tumour stage and SLIT2 methylation status: SLIT2 methylation was present in 33% of stage 1, 2 and 4S tumours and in 26% of stage 3 and 4 tumours ($P = 0.73$).

**Methylation analysis of SLIT2 in primary Wilms’ tumours**

Next, we proceeded to analyse SLIT2 promoter methylation status in 37 Wilms’ tumours that had been investigated previously for RASSF1A and CASP8 promoter methylation status (Wagner et al, 2002 and unpublished observations). In total, 38% (14 out of 37) Wilms’ tumours demonstrated SLIT2 CpG island promoter methylation (Figure 1B). Promoter CpG island methylation was confirmed by direct sequencing in one tumour (Figure 4B). All SLIT2 methylated tumours contained unmethylated SLIT2 alleles that might be attributable to the presence of contaminating normal tissue (tumour samples were not microdissected). SLIT2 methylation was detected in zero of six normal tissue samples corresponding to the methylated tumours.

To investigate the 4p15.2 allelic status of Wilms’ tumours with SLIT2 methylation, we typed six methylated tumours for LOH at SLIT2, SK-N-SH and other cancer genes in primary Wilms’ tumours

**SLIT2 and other cancer genes in primary Wilms’ tumours**

To investigate the relationships between SLIT2 promoter methylation and de novo methylation of RASSF1A and CASP8, we compared the frequencies of CASP8 and RASSF1A methylation in tumours with and without SLIT2 methylation. In tumours with SLIT2 methylation, CASP8 and RASSF1A were methylated in 43% (six out of 14) and 36% (five out of 14), respectively. In tumours without SLIT2 methylation, CASP8 and RASSF1A promoter methylation was detected in 39% (nine out of 23) and 70% (16...
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The frequency of relapse in Wilms’ tumours with SLIT2 methylation was similar to that without SLIT2 methylation (21% (three out of 14) and 17% (four out of 23), respectively), and there was no significant association between SLIT2 methylation and advanced stage tumours (the frequency of stage 3 and 4 tumours in the SLIT2 methylated and unmethylated groups was 45% (five out of 11) and 63% (12 out of 19), respectively).

Methylation analysis of SLIT2 in primary RCC

We detected SLIT2 promoter methylation in 25% (12 out of 48) primary RCC and in 75% (six out of eight) RCC cell lines (Figure 1C). Promoter CpG island methylation was confirmed by direct sequencing of five clones from RCC cell lines 2 and 3 tumours (Figure 4A). SLIT2 promoter methylation was also detected in one out of 12 of the matching normal kidney tissue samples for methylated tumours. All RCC with SLIT2 methylation also contained unmethylated SLIT2 alleles, which might be attributable to the presence of contaminating normal tissue (tumour samples were not microdissected). Loss of heterozygosity at D4S1546 was not detected in 10 informative RCC with SLIT2 methylation.

SLIT2 promoter methylation is associated with transcriptional silencing in an RCC cell line We investigated the possible association between the SLIT2 promoter region CpG island methylation and SLIT2 transcript expression in a panel of RCC cell lines (SKRC 18, SKRC 39, SKRC 45, SKRC 47, SKRC 54, KTCL 26, UMRC-2 and 786-0). Cells were treated with the demethylating agent 5-aza-dC for 5 days. Except for SKRC 45 and SKRC 47 (both unmethylated for SLIT2), SLIT2 expression was significantly increased in the kidney tumour cell lines after 5-aza-dC treatment. GAPDH expression levels were equal in both 5-aza-dC-treated and untreated cell lines (Figure 3).

Methylation status of SLIT2 and inactivation of RASSF1A and VHL in primary RCC Previously, we analysed primary RCC for RASSF1A methylation and inactivation of the VHL tumour suppressor gene. There was no association between SLIT2 methylation and the presence of VHL mutation in clear cell RCC, and the frequency of RASSF1A methylation was similar in RCC with and without SLIT2 methylation (25 and 21%, respectively).

SLIT2 methylation status and clinicopathological status The frequency of SLIT2 methylation in clear cell RCC (24%, nine out of 37) was similar to that found in all tumour types. There was no significant association between SLIT2 methylation status and grade or TNM status.

DISCUSSION

Previously, we (a) identified SLIT2 promoter methylation in 53% non-small-cell lung cancer, 36% small-cell lung cancer and 43% of breast cancers, (b) demonstrated that promoter methylation is associated with reversible transcriptional silencing and (c) determined that restoration of SLIT2 expression suppressed tumour growth in in vitro studies (Dallol et al, 2002a).

Thus having established SLIT2 as a lung and breast cancer suppressor gene, we have now identified frequent SLIT2 hypermethylation in paediatric cancers and in RCC and, recently, in 59% of gliomas (Dallol et al, 2003). Slit protein binds to the roundabout receptor and acts as a midline repellent to guide axonal development during embryogenesis (Rajagopalan et al, 2000; Simpson et al, 2000; Wong et al, 2001) In humans, three SLIT orthologues have been identified, but to date only SLIT2 has been implicated in cancer. In mice, Robo1 inactivation produces delayed lung maturation and bronchial hyperplasia (Xian et al, 2001) and we have demonstrated de novo ROBO1 promoter methylation in 19% primary invasive breast carcinomas and 18% primary clear cell RCC, although somatic mutations were not identified (Dallol et al, 2002b).

The characterisation of tumour-specific TSG methylation profiles provides insights into the molecular pathology of the tumour and can be used to establish tumour-specific methylation profiles (Esteller et al, 2001a). It is anticipated that such profiles will be useful for diagnosis and clinicopathological classification. In colorectal cancer, the identification of a subset of tumours with widespread de novo methylation of TSG and mismatch repair genes (‘the methylator phenotype’) (Toyota et al, 1999) has been of considerable interest and it is unclear as to whether similar subtypes will be a feature of other cancers.

The molecular pathology of sporadic NB has been investigated extensively by molecular cytogenetic and LOH analysis. Frequent alterations include N-myc amplification (20–25%) and gain of genetic material at 17q23–qter (~50% of tumours). Neuroblas-
toma TSGs have been mapped by LOH studies to 1p36 (~35% in primary tumours show LOH), 11q23 (44%), 14q23–qter (22%) and 3p (15%) (Ejeskar et al, 1998; Maris and Matthay, 1999). However, specific gene mutations have not been defined, although we did identify RASSF1A methylation in 55% of NBs and that CASP8 methylation occurs in ~50% of tumours (Teitz et al, 2000; Astuti et al, 2001). Caron et al (1996) reported 4p allele loss in ~20% of NB and we have now identified promoter methylation of the 4p15.2 candidate TSG SLIT2 in 29% of NBs. Thus, epigenetic inactivation of SLIT2 is a common feature of NB, although less frequent than methylation of CASP8 and RASSF1A. Harada et al (2002b) reported that NB patients with CASP8 methylation were older than those without tumour methylation, but to date, CASP8, RASSF1A or SLIT2, APC, GSTP1, CDH1 and CDH13 are rarely methylated in NB (Harada et al, 2002a).

Although de novo methylation and silencing of H19 in Wilms' tumours was first reported some years ago (Steenman et al, 1994; Taniguchi et al, 1995), epigenetic changes have not been investigated in detail in Wilms' tumour. Recently, we reported frequent CASP8 (43%) and RASSF1A (56%) promoter methylation in Wilms' tumours and the present study has demonstrated that SLIT2 methylation represents a further frequent epigenetic change in Wilms' tumours. To date, we have not identified an association between CASP8, RASSF1A and SLIT2 methylation in individual tumours, so there is little evidence of a methylator phenotype in a subset of Wilms' tumours. Indeed there was a negative, albeit statistically insignificant, correlation between H19 methylation and silencing of the 4p15.2 candidate TSG which may be implicated in early tumorigenesis. In tumours such as colorectal cancer, where there is a well-validated adenoma–carcinoma sequence, it is possible to define the genetic changes associated with different stages of tumorigenesis. However, in sporadic cases of NB, Wilms' tumour and RCC, there is generally no well-defined pathway from precursor lesion to tumour (although nephroblastomatosis may be present in patients with Beckwith–Wiedemann syndrome and 'early lesion RCC' has been described in von Hippel-Lindau disease). Hence, we are unable to precisely define when SLIT methylation occurs in the pathogenesis of these tumours. However, it is known that TSG inactivation may be an early event in tumorigenesis. Thus, methylation may be the 'second hit' in familial cancer syndrome tumours (Prowse et al, 1997, Esteller et al, 2001b). Furthermore, in sporadic and familial adenomatous polyposis coli, TSG CpG island methylation may be detected in the earliest precursor lesion in colorectal carcinogenesis, aberrant crypt foci (Chan et al, 2002). Similarly, Zöchbauer-Müller et al (2001) suggested that TSG methylation may be a presneoplastic change in non-small-cell lung cancer. We have analysed previously RASSF1A promoter methylation status in normal, duodenal-carcinoma-in-situ (DCIS) and breast cancer trios. RASSF1A promoter hypermethylation was detected in 65% of invasive cancers and in 42% of corresponding DCIS but in none of the normal breast samples (Honorio et al, 2003). In all, 30% of DCIS without invasive breast cancer also underwent RASSF1A promoter hypermethylation, suggesting that inactivation of RASSF1A by CpG island methylation is an early event in breast tumorigenesis. Preliminary unpublished data also reveal SLIT2 methylation in DCIS samples (RE Dickinson and F Latif, unpublished). Thus, there is evidence that SLIT2 hypermethylation can be implicated in early tumorigenesis.

In breast and lung cancers, TSG promoter methylation SLIT2 appears to resemble TSGs such as RASSF1A, as epigenetic inactivation is more frequent than somatic mutations. RASSF1A methylation has been reported in a wide range of human cancers. We have demonstrated that SLIT2 methylation is common in paediatric and adult cancers, and further analysis of additional tumour types seems indicated. Frequent 4p allele loss has been reported in cancers that demonstrate SLIT2 methylation such as lung, breast and NB, and also in cancers in which SLIT2 methylation status has not been investigated including colorectal, bladder and head and neck cancers (Knowles et al, 1994; Rosin et al, 1995; Gryfe et al, 1997; Pershouse et al, 1997; Yustein et al, 1999; Girard et al, 2000). During development, the SLIT2 protein functions as a secreted chemorepellent so that restoration of SLIT2 function by reversal of epigenetic inactivation or administration of SLIT2 agonists might provide novel therapeutic opportunities for human cancers.

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