Epigenetic analysis of HIC1, CASP8, FLIP, TSP1, DCR1, DCR2, DR4, DR5, KvDMR1, H19 and preferential 11p15.5 maternal-allele loss in von Hippel-Lindau and sporadic phaeochromocytomas

C D E Margetts¹, D Astuti¹, D C Gentle¹,², W N Cooper¹, A Cascon³, D Catchpoole⁴, M Robledo³, H P H Neumann⁵, F Latif¹,² and E R Maher¹,²

¹Section of Medical and Molecular Genetics, Department of Paediatrics and Child Health, University of Birmingham, The Medical School, Edgbaston, Birmingham B15 2TT, UK
²Cancer Research UK Renal Molecular Oncology Research Group, University of Birmingham, The Medical School, Edgbaston, Birmingham B15 2TT, UK
³Hereditary Endocrine Cancer Group, Department of Human Genetics, Centro Nacional de Investigaciones Oncologicas, Madrid, Spain
⁴The Tumour Bank, The Children’s Hospital at Westmead, Locked Bag 4001, Westmead, NSW, 2145, Australia
⁵Department of Nephrology, Albert-Ludwigs-University, Freiburg, Germany

(Requests for offprints should be addressed to E R Maher; Email: E.R.Maher@bham.ac.uk)

Abstract

Phaeochromocytoma is a neural-crest-derived tumour that may be a feature of several familial cancer syndromes including von Hippel-Lindau (VHL) disease, multiple endocrine neoplasia type 2 (MEN 2), neurofibromatosis type 1 (NF1) and germline succinate dehydrogenase subunit (SDHB and SDHD) mutations. However the somatic genetic and epigenetic events that occur in phaeochromocytoma tumourigenesis are not well defined. Epigenetic events including de novo promoter methylation of tumour-suppressor genes are frequent in many human neoplasms. As neuroblastoma and phaeochromocytoma are both neural-crest-derived tumours, we postulated that some epigenetic events might be implicated in both tumour types and wished to establish how somatic epigenetic alterations compared in VHL-associated and sporadic phaeochromocytomas. We identified frequent aberrant methylation of HIC1 (82%) and CASP8 (31%) in phaeochromocytoma, but both genes were significantly more methylated in VHL phaeochromocytomas than in sporadic cases. Of four tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors analysed, DR4 was most commonly methylated (41%; compared with DrC2 (26%), DrC1 (23%) and DR5 (10%)). Gene methylation patterns in phaeochromocytoma and neuroblastoma did not differ significantly suggesting overlapping mechanisms of tumourigenesis. We also investigated the role of 11p15.5-imprinted genes in phaeochromocytoma. We found that in 10 sporadic and VHL phaeochromocytomas with 11p15.5 allele loss, the patterns of methylation of 11p15.5-differentially methylated regions were consistent with maternal, rather than, paternal chromosome loss in all cases (P<0.001). This suggests that 11p15.5-imprinted genes may be implicated in the pathogenesis of both familial (germline VHL and SDHD mutations) and sporadic phaeochromocytomas.

Endocrine-Related Cancer (2005) 12 161–172

Introduction

Phaeochromocytomas are catecholamine-producing tumours that usually arise within the adrenal medulla but are extra-adrenal in 10% of cases. Hypertension is the most consistent clinical feature of phaeochromocytoma and cardiovascular disease is the leading cause of death, but about 10% are malignant. As with many human neoplasms, a subset of phaeochromocytomas occur in genetically susceptible individuals.
Thus inherited phaeochromocytoma may be a feature of von Hippel-Lindau (VHL) disease, multiple endocrine neoplasia type 2 (MEN 2), neurofibromatosis type 1 (NF1) and germline succinate dehydrogenase (SDH) subunit mutations (Maher & Eng 2002, Neumann et al. 2002). However, somatic mutations in VHL, RET, SDHB and SDHD are rare in sporadic phaeochromocytomas. Thus Bender et al. (Teitze et al. 1996, Hofstra et al. 1998, Maris & Matthay 1999). Frequent epigenetic alterations in neuroblastoma include CASP8 and RASSF1A hypermethylation (Teitz et al. 2000, Astuti et al. 2001c). RASSF1A promoter methylation also occurs in approximately 20% of phaeochromocytomas so it appears that there may be an overlap between the genetic and epigenetic events implicated in neuroblastoma and phaeochromocytoma. To further investigate the mechanisms of tumourigenesis in sporadic and familial phaeochromocytoma we have investigated the epigenetic status of FLIP, TSP1, DcR1, DcR2, DR4, DR5, CASP8 and HIC1 genes in VHL and sporadic phaeochromocytomas. In addition in view of the observation that preferential maternal chromosome 11 allele loss in phaeochromocytomas associated with inherited SDHD mutations is apparently not related to SDHD imprinting (Hensen et al. 2004), we investigated the parent-of-origin effects on 11p15.5-allele-loss VHL and sporadic phaeochromocytomas.

Patients and methods

Patients and samples

For the analysis of HIC1, CASP8, FLIP, TSP1, DcR1, DcR2, DR4 and DR5 a total of 59 tumour samples were analysed (20 neuroblastomas, 23 VHL-associated phaeochromocytomas and 16 sporadic phaeochromocytomas). In the 11p15.5 LOH study, a total of 48 samples were analysed (40 sporadic and eight VHL-associated phaeochromocytomas). Informed consent and approval from the appropriate Institutional Review Boards were obtained for all samples. DNA was extracted from tumour and normal tissue (blood or matched kidney) by standard methods.

Cell lines

Eight neuroblastoma cell lines were used: SK-N-AS, SK-N-F1, SK-N-DZ, SK-N-MC, SK-N-BE, SK-N-SH (ATCC, Manassas, VA, USA), KELLY and CHP212.

Sodium bisulphite modification

Sodium bisulphite modification was carried out using an adapted method (Herman et al. 1996). Genomic DNA (0.5–1.0μg) was denatured at 37 °C for 10 min in 0.3 M NaOH. Unmethylated cytosines were sulphonated by incubation in 3.12 M sodium bisulphite/1 M hydroquinone (pH 5) at 95 °C for 10 s and then 50 °C for 15 min for 20 cycles. The resulting sulphonated DNA was purified using the Wizard DNA cleanup 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 NaOH for 5 min at room temperature, then the DNA was precipitated with sodium acetate (5 μl, 3 M) and ethanol (125 μl, 100%) overnight at −20 °C and resuspended in 50 μl distilled water.

Methylation-specific PCR (MSP)

MSP was performed essentially using previously published primers and conditions (Dong et al. 2001, van Noesel et al. 2002, 2003, Yang et al. 2003). Expected PCR products are as follows: for DcR1, 125 (methylated)/135 (unmethylated) bp; DcR2, 138 (methylated)/145 (unmethylated) bp; DR4, 91 (methylated)/102 (unmethylated) bp; DR5, 199 (methylated)/208 (unmethylated) bp; CASP8, 321 (methylated)/322 (unmethylated) bp; FLIP, 203 (methylated)/211 (unmethylated); TSP1, 74 (methylated)/115 (unmethylated) bp; HIC1, 95 (methylated)/118 (unmethylated) bp.

Reactions were hot-started at 95 °C for 15 min, by using 0.25 μl HotstarTaq DNA polymerase (Qiagen; 5 units/μl) PCR products were visualized on 2% agarose gels stained with ethidium bromide. Genomic
DNA methylated in vitro using SsSI methylase (New England Biolabs) was used as a positive control for MSP.

**Treatment of cell lines with 5-aza-2'-deoxycytidine (5-aza-dC)**

5-aza-dC (Sigma) was prepared freshly in double-distilled H2O at 2 mg/ml and filter-sterilized. 1 × 10^6 cells were plated in 75 cm² flasks in RPMI 1640 medium supplemented with 10% fetal calf serum and left to settle for 24 h (day 0). Cells were treated with 2μM 5-aza-dC on days 1 and 4 and harvested on day 5. The culture medium was changed before each treatment and 24 h after treatment. Total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer’s guidelines.

**Expression analysis**

Gene expression was detected by reverse transcriptase PCR. 1μg RNA was reverse transcribed using Reverse Transcription Systems and oligo dT primers (Promega) according to the manufacturer’s protocols. 1 μl of the cDNA obtained was then used as a template for PCR amplification. Primer sequences and conditions were described previously (Dong et al. 2001, van Noesel et al. 2002, 2003, Yang et al. 2003). Expected PCR products are as follows: DcR1, 242 bp; DcR2, 243 bp; DR4, 222 bp; TSP1, 159 bp. As a control, the GAPDH primers used were 5'-AAGGTGAAGTCTGACAC-3' and 5'-CAGCCTTCTCCAATGTGTG-3', resulting in a 242 bp PCR product of 319 bp. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

**Analysis of 11p15.5 maternal-allele loss**

Chromosome 11p15.5 LOH was assessed by DNA microsatellite analysis with tyrosine hydroxylase (TH) using primers 5'-GATTCC-3' (forward) and 5'-TCTTACTAAAAACTCCCTAAAAATC-3' (reverse), resulting in a 301 bp fragment, and H19 amplified for KvDMR1 using primers 5'-GGGAGGCACCAACAGATG-3' (forward) and 5'-CTTTTGGTAGTGTAGGTGGTTTATG-3' (reverse), resulting in 213 bp PCR products. Amplified products were then digested with BsUI restriction enzyme, run in an ABI 3730 or 377 DNA analyser with LIZ-500 size standards and analysed with Genemapper v.3 software (WN Cooper & ER Maher, unpublished observation). The methylated allele undergoes digestion to generate two fragments while in the unmethylated allele the recognition site is lost following bisulphite modification and is therefore not cleaved. The methylation index (MI) was calculated as M/(M+U) in which Mis the peak area of the methylated allele and U is the peak area of the unmethylated allele.

**Statistical analysis**

Fisher’s exact test, the binomial distribution and analysis of variance (ANOVA) were used as appropriate. P values of <0.05 were taken as statistically significant.

**Results**

**FLIP, TSP1, DcR1, DcR2, DR4, DR5, CASP8 and HIC1 methylation status in primary pheochromocytomas and neuroblastomas**

We used MSP analysis to investigate the methylation status of eight genes in 39 pheochromocytomas (23 VHL-related and 16 sporadic) and 20 neuroblastoma tumours (see Fig. 1). The results are summarized in Table 1 and individual tumour results are given in Table 2 (all methylation-positive tumours also demonstrated unmethylated alleles). The frequency of gene methylation varied from 8% (FLIP) to 82% (HIC1), with methylation in five genes exceeding 20% (HIC1, CASP8, DcR1, DcR2 and DR4). For neuroblastoma the frequency of gene methylation varied from 0% (DR5) to 56% (HIC1), with methylation in six genes exceeding 20% (TSP1, HIC1, CASP8, DcR1, DcR2 and DR4). Mean (±s.d.) MI values (number of genes methylated/number of genes tested) were 0.35±0.15 for VHL pheochromocytomas, 0.23±0.15 for sporadic...
phaeochromocytomas and 0.325 ± 0.22 for neuroblastoma and there was no evidence of significant differences in MI between the three tumour types ($F = 2.27$, $P = 0.11$). When each of the eight genes were considered separately there were no significant differences between the frequency of methylation in neuroblastomas and phaeochromocytomas ($P > 0.2$). However, the frequency of methylation of $HIC1$ and $CASP8$ was significantly higher in VHL phaeochromocytomas than in sporadic phaeochromocytomas (100 versus 56%, $P = 0.001$; 48 versus 6%, $P = 0.006$). The frequency of $HIC1$ methylation in VHL phaeochromocytoma was higher than in neuroblastoma (100 versus 80%, $P = 0.039$), and the frequency of $CASP8$ methylation in sporadic phaeochromocytoma was significantly lower than in neuroblastoma (6 versus 40%, $P = 0.023$).

Considering all tumours analysed there was a significant association between $HIC1$ and $CASP8$ methylation ($P < 0.05$), and $CASP8$ methylation was only

**Figure 1** MSP of $FLIP$, $TSP1$, $HIC-1$, $CASP8$, $DcR1$, $DcR2$, $DR4$ and $DR5$ in neuroblastoma (NB) and phaeochromocytoma (PV) tumours. Bisulphite-modified DNA was amplified with primers specific for unmethylated (U) and methylated (M) DNA. Sample numbers are indicated above the bands. Sizes of the PCR products are indicated with arrows on the right; unmethylated and methylated respectively.

**Table 1** Summary of gene-specific methylation data in VHL-associated and sporadic phaeochromocytomas and neuroblastoma tumours and cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Proportion of tumours methylated (%)</th>
<th>Tumours methylated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>VHL</td>
<td>Sporadic</td>
</tr>
<tr>
<td>$TSP1$</td>
<td>23% (9/39)</td>
<td>22% (5/23)</td>
</tr>
<tr>
<td>$HIC1$</td>
<td>82% (32/39)</td>
<td>100% (23/23)**</td>
</tr>
<tr>
<td>$FLIP$</td>
<td>8% (3/39)</td>
<td>13% (3/23)</td>
</tr>
<tr>
<td>$CASP8$</td>
<td>32% (12/38)</td>
<td>48% (11/23)*</td>
</tr>
<tr>
<td>$DcR1$</td>
<td>24% (9/38)</td>
<td>17% (4/23)</td>
</tr>
<tr>
<td>$DcR2$</td>
<td>26% (10/39)</td>
<td>35% (8/23)</td>
</tr>
<tr>
<td>$DR4$</td>
<td>41% (16/39)</td>
<td>39% (9/23)</td>
</tr>
<tr>
<td>$DR5$</td>
<td>10% (4/39)</td>
<td>9% (2/23)</td>
</tr>
</tbody>
</table>

**$P = 0.001$.**

* $P = 0.023$. **
<table>
<thead>
<tr>
<th>Tumour type and number</th>
<th>FLIP</th>
<th>TSP1</th>
<th>DcR1</th>
<th>DcR2</th>
<th>DR4</th>
<th>DR5</th>
<th>CASP8</th>
<th>HIC1</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV01</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV02</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV03</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV04</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV05</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV06</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV07</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV08</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV09</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV10</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV11</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV12</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV13</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV14</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV15</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV16</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV17</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV18</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV19</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV20</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PV21</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV22</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PV23</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PS01</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PS02</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PS03</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PS04</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS05</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PS06</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS07</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PS08</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS09</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS10</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS11</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PS12</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS13</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS14</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS15</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>PS16</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB01</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB02</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB03</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB04</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB05</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB06</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB07</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB08</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB09</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB10</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB11</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB12</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
</tr>
<tr>
<td>NB13</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB14</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB15</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB16</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB17</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB18</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB19</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>NB20</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>U</td>
<td>U</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
</tbody>
</table>

NB, neuroblastoma; PS, sporadic phaeochromocytoma; PV, VHL phaeochromocytoma; U, unmethylated; M, methylated.
detected in tumours with HIC1 methylation. A significant association \( (P < 0.05) \) was still detected after excluding VHL phaeochromocytomas from the analysis. No other significant correlations were detected between methylation of specific genes apart from an association between CASP8 and DCR2 methylation \( (P = 0.035) \).

**Correlation between gene methylation and expression in neuroblastoma cell lines**

To confirm the functional significance of gene methylation, we analysed eight neuroblastoma cell lines for methylation status at FLIP, TSP1, DcR1, DcR2, DR4 and DR5. Methylation at FLIP and DR5 was not detected in any of the cell lines but for the rest at least partial methylation was detected in two or more cell lines \( (TSP1 = 3/8, \ DcR1 = 6/8, \ DcR2 = 8/8 \text{ and } DR4 = 2/8) \). We then determined the effect of treatment with 5-aza-dC on transcription of four methylated genes \( (DcR1, \ DcR2, \ DR4 \text{ and } TSP1) \) in two neuroblastoma cell lines (SK-N-DZ and SK-N-SH; see Fig. 2). In each case demethylation increased expression.

**Epigenetic and genetic analysis of the 11p15.5-imprinted gene cluster**

26 phaeochromocytomas (20 sporadic and six VHL tumours) were informative for 11p15.5 allele loss at the TH locus. 35% \( (7/20) \) of sporadic and 50% \( (3/6) \) VHL phaeochromocytomas demonstrated LOH (Table 3).

Sufficient DNA was available to analyse the methylation status of two 11p15.5 DMRs, KvDMR1 (maternal allele methylated) and H19 CTCF upstream region (paternal allele methylated), in 15 phaeochromocytomas without 11p15.5 LOH and 10 tumours with 11p15.5 LOH. In one of the 15 phaeochromocytomas without 11p15.5 allele loss (tumour PS14), no methylation was detected at KvDMR1, but the H19 DMR MI was normal (see Table 3). In the remaining 14 phaeochromocytomas without allele loss, mean \( \pm S.D. \) KvDMR1 MI was \( 0.217 \pm 0.12 \) and mean H19 DMR MI was \( 0.343 \pm 0.05 \). In each of these tumours the H19 DMR MI/KvDMR1 MI ratio was \( < 3 \). In contrast, the ratio of H19 DMR MI to KvDMR1 MI was \( > 3 \) in all phaeochromocytomas with 11p15.5 allele loss (mean \( \pm S.D. \) : KvDMR1 MI = \( 0.096 \pm 0.04 \) and H19 DMR MI = \( 0.478 \pm 0.1 \).

These findings were consistent with maternal-allele loss (paternal-allele loss would result in a high MI\( _{KvDMR1} \) and a low MI\( _{H19} \)) in all cases and, assuming equal probability of paternal- and maternal-allele loss, these results were significant \( (P = 0.001) \). Both VHL-associated \( (n = 3) \) and sporadic phaeochromocytomas \( (n = 7) \) demonstrated preferential maternal-allele loss. Figure 3 shows representative samples with LOH at the TH locus and loss of KvDMR1 methylation and retention of heterozygosity at TH and normal H19 and KvDMR1 methylation.

**Discussion**

Phaeochromocytoma and neuroblastoma are both derived from the neural crest, yet phaeochromocytoma-susceptibility genes (e.g. VHL, RET, NF1, SDHB and SDHD) have not been implicated in familial neuroblastoma and PHOX2B mutations have been associated with familial neuroblastoma but not phaeochromocytoma (Maher & Eng 2002, De Preter et al. 2004, Trochet et al. 2004) However overlap between regions of allele loss in the two tumours suggests that there could be similarities in the somatic genetic and epigenetic events involved in the pathogenesis of the two tumour types. Hence, in order to compare somatic epigenetic events we analysed the methylation status of eight genes implicated previously in neuroblastoma tumour development \( (FLIP, \ TSP1, \ DcR1, \ DcR2, \ DR4, \ DR5, \ CASP8 \text{ and } HIC1) \). Previously we had demonstrated promoter methylation of the RASSF1A tumour-suppressor gene in 22% of sporadic phaeochromocytomas and 55% of
neuroblastomas (Astuti et al. 2001c), suggesting that analysis of genes implicated in neuroblastoma tumourigenesis might provide insights into the pathogenesis of phaeochromocytoma, but HIC1, FLIP, TSP1, DcR1, DcR2, DR4, DR5 and CASP8 had not been analysed previously in phaeochromocytomas.

The hypermethylated in cancer-1 gene (HIC1) encodes a POZ-family zinc-finger transcription factor. Promoter methylation and transcriptional silencing of HIC1 candidate tumour-suppressor gene has been reported in many paediatric and adult cancers and germline heterozygous disruption of HIC1 in mice results in cancer susceptibility (mostly epithelial cancers in males and lymphomas and sarcomas in females; Chen et al. 2003), including neuroblastomas (Rathi et al. 2003). We have now demonstrated frequent HIC1 methylation in phaeochromocytomas, so that HIC1 methylation is not restricted to malignant tumours.

Previously chromosome 11 and 3p allele loss was associated preferentially with VHL phaeochromocytomas whereas 1p allele loss was significantly more common in sporadic phaeochromocytomas (Bender et al. 2000, Lui et al. 2002). The differential methylation of HIC1 and CASP8 in VHL phaeochromocytoma compared with sporadic tumours extends the observation of differential regions of allele loss and provides the first links to specific genes.

Tumour angiogenesis, a major requirement for tumour outgrowth and metastasis, is regulated by pro- and anti-angiogenic factors. VHL inactivation leads to upregulation of HIF-1 and HIF-2 transcription factors and a consequent increase in hypoxia-inducible genes including angiogenic factors such as vascular endothelial growth factor (VEGF; Maxwell et al. 1999). Both VHL-associated and sporadic phaeochromocytomas are vascularized tumours, although the relevance of HIF dysregulation for the development of phaeochromocytoma in VHL disease is unclear.

### Table 3 Methylation index (MI) at H19 DMR and at KvDMR1 (and ratio of the two MIs) in phaeochromocytomas with (LOH) and without (RET) allele loss at 11p15.5

<table>
<thead>
<tr>
<th>Tumour ID</th>
<th>11p15.5 status</th>
<th>11q23.3 status</th>
<th>H19 DMR MI</th>
<th>KvDMR1 MI</th>
<th>H19/KvDMR1 MI ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS21</td>
<td>LOH</td>
<td>NI</td>
<td>0.67</td>
<td>0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>PS14</td>
<td>RET</td>
<td></td>
<td>0.35</td>
<td>0</td>
<td>&gt;10</td>
</tr>
<tr>
<td>PS25</td>
<td>LOH</td>
<td>RET</td>
<td>0.54</td>
<td>0.07</td>
<td>7.7</td>
</tr>
<tr>
<td>PS07</td>
<td>LOH</td>
<td>NI</td>
<td>0.6</td>
<td>0.09</td>
<td>6.7</td>
</tr>
<tr>
<td>PV16</td>
<td>LOH</td>
<td>LOH</td>
<td>0.46</td>
<td>0.08</td>
<td>5.8</td>
</tr>
<tr>
<td>PV07</td>
<td>LOH</td>
<td>NI</td>
<td>0.4</td>
<td>0.08</td>
<td>5</td>
</tr>
<tr>
<td>PS30</td>
<td>LOH</td>
<td>NI</td>
<td>0.42</td>
<td>0.11</td>
<td>3.8</td>
</tr>
<tr>
<td>PV23</td>
<td>LOH</td>
<td>NI</td>
<td>0.37</td>
<td>0.1</td>
<td>3.7</td>
</tr>
<tr>
<td>PS29</td>
<td>LOH</td>
<td>LOH</td>
<td>0.48</td>
<td>0.13</td>
<td>3.7</td>
</tr>
<tr>
<td>PS18</td>
<td>LOH</td>
<td>LOH</td>
<td>0.39</td>
<td>0.15</td>
<td>3.3</td>
</tr>
<tr>
<td>PS24</td>
<td>LOH</td>
<td>RET</td>
<td>0.45</td>
<td>0.15</td>
<td>3</td>
</tr>
<tr>
<td>PS32</td>
<td>RET</td>
<td></td>
<td>0.41</td>
<td>0.15</td>
<td>2.7</td>
</tr>
<tr>
<td>PS05</td>
<td>RET</td>
<td></td>
<td>0.33</td>
<td>0.12</td>
<td>2.7</td>
</tr>
<tr>
<td>PV22</td>
<td>RET</td>
<td></td>
<td>0.36</td>
<td>0.15</td>
<td>2.4</td>
</tr>
<tr>
<td>PS27</td>
<td>RET</td>
<td></td>
<td>0.31</td>
<td>0.19</td>
<td>2.4</td>
</tr>
<tr>
<td>PS34</td>
<td>RET</td>
<td></td>
<td>0.28</td>
<td>0.14</td>
<td>2</td>
</tr>
<tr>
<td>PS41</td>
<td>RET</td>
<td></td>
<td>0.37</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td>PV15</td>
<td>RET</td>
<td></td>
<td>0.33</td>
<td>0.18</td>
<td>1.8</td>
</tr>
<tr>
<td>PV19</td>
<td>RET</td>
<td></td>
<td>0.29</td>
<td>0.23</td>
<td>1.3</td>
</tr>
<tr>
<td>PS06</td>
<td>RET</td>
<td></td>
<td>0.45</td>
<td>0.37</td>
<td>1.2</td>
</tr>
<tr>
<td>PS33</td>
<td>RET</td>
<td></td>
<td>0.45</td>
<td>0.41</td>
<td>1.1</td>
</tr>
<tr>
<td>PS12</td>
<td>RET</td>
<td></td>
<td>0.32</td>
<td>0.32</td>
<td>1.0</td>
</tr>
<tr>
<td>PS40</td>
<td>RET</td>
<td></td>
<td>0.31</td>
<td>0.34</td>
<td>0.9</td>
</tr>
<tr>
<td>PS17</td>
<td>RET</td>
<td></td>
<td>0.33</td>
<td>0.39</td>
<td>0.8</td>
</tr>
</tbody>
</table>

NI, non informative.
(Clifford et al. 2001, Hoffman et al. 2001), so other events may promote angiogenesis. Thrombospondin-1 (TSP1 or THBS1) is a potent anti-angiogenic factor and inhibits a variety of angiogenic stimuli. Aberrant methylation of TSP1 has been detected in a variety of neoplasms including gastric cancers (Oue et al. 2003) and carcinoïd tumours (Chan et al. 2003). TSP1 promoter methylation has been reported in 37% of neuroblastomas and is associated with loss of expression in neuroblastoma cell lines (Yang et al. 2003). In our series aberrant methylation of TSP1 was detected in 30% of neuroblastoma and 18% of phaeochromocytomas, consistent with the hypothesis that this will promote tumourigenesis in a subset of these neural-crest-derived tumours.

CASP8, FLIP, DCR1, DCR2, DR4 and DR5 are all implicated in apoptotic pathways. Apoptosis in mammalian cells can be initiated through two major interrelated pathways, one involving engagement of the tumour necrosis factor (TNF) family of death receptors, the other involving the release of cytochrome c from mitochondria. TNF-related apoptosis-inducing ligand (TRAIL) preferentially induces apoptosis in tumour cell lines and CASP8 methylation and transcriptional silencing leads to resistance to TRAIL (Eggert et al. 2001). FLIP (FLICE-like inhibitory protein) is a negative regulator of CASP8. DCR1, DCR2, DR4 and DR5 are receptors for TRAIL, but while DR4 and DR5 contain cytoplasmic death domains and are pro-apoptotic, DcR1 and DcR2 are decoy receptors that lack a functional death domain and do not mediate TRAIL-induced apoptosis. In addition to their related functions, CASP8 and FLIP co-localize to chromosome 2q33 and DcR1, DcR2, DR4 and DR5 to chromosome 8p21. Although van Noesel et al. (2003) found correlations between methylation of pairs of co-located genes (i.e. CASP8 and FLIP, DCR1 and DCR2, and DR4 and DR5) in neuroblastoma, these associations were not replicated in our combined dataset of all neuroblastomas and phaeochromocytomas. Furthermore Shivapurkar et al. (2004) also found independent methylation of the four TRAIL receptor genes in human cancers. Methylation of CASP8, DR4 or DR5 would be predicted to reduce sensitivity to

![Figure 3](A) LOH at the TH locus and loss of methylation at KvDMR1 in tumour PS07 and (B) retention of heterozygosity at the TH locus and normal H19 and KvDMR1 methylation in tumour PS17. Boxes: upper label indicates the length of the fragment (H19, 132 bp methylated paternal allele and 221 bp unmethylated maternal allele; KvDMR1, 265 bp methylated maternal allele and 305 bp unmethylated paternal allele) and the lower label indicates the peak area.
TRAIL apoptosis and methylation of at least one or more of these genes was detected in 67% of phaeochromocytomas and 55% of neuroblastomas. Although DcR1 and DcR2 do not mediate TRAIL-induced apoptosis, their frequent methylation and transcriptional silencing in paediatric and adult cancers does suggest that inactivation promotes tumorigenesis. In this context it is interesting to note that TRAIL can activate the anti-apoptotic nuclear factor kB (NF-κB) pathway through DR4 and DR5. On the other hand, DcR1 and DcR2 are thought to inhibit TRAIL-induced NF-κB activation via DR4 and DR5, so according to this model methylation and downregulation of DcR1 and DcR2 would be pro-tumourigenic (Shivapurkar et al. 2004).

Germline SDHD mutations cause phaeochromocytoma and head and neck paraganglioma susceptibility with parent-of-origin effects such that disease only develops after paternal transmission (van der Mey et al. 1989, Baysal et al. 2000, Astuti et al. 2001a). However, no convincing evidence for genomic imprinting of SDHD has been reported. Recently it was suggested that both biallelic SDHD inactivation and loss of maternally expressed 11p15.5 tumour-suppressor genes might be required for the development of SDHD-associated phaeochromocytoma (Hensen et al. 2004). For many adult-onset tumours, parental samples are not available to allow direct parent-of-origin effects on allele loss to be investigated. Hence we took the indirect approach of studying the methylation status of two differentially methylated regions in 11p15.5. As generalized genome hypomethylation may be a feature of some human cancers, we compared the ratios of methylation indices at KvDMR1 and H19 DMR. In all phaeochromocytomas with 11p15.5 allele loss there was increased H19 to KvDMR1 methylation consistent with maternal-allele loss and no evidence of the pattern expected with paternal-allele loss (reduced H19 and increased KvDMR1 methylation). Thus our findings suggest that preferential maternal 11p15.5 allele loss is not restricted to phaeochromocytomas with germline SDHD mutations, but also occurs in VHL and sporadic phaeochromocytomas. This finding strengthens the interpretation that SDHD is not imprinted and that preferential maternal chromosome 11 allele loss reflects the involvement of 11p15.5-imprinted genes in tumourigenesis. Somatic SDHD mutations have not been reported in sporadic phaeochromocytomas (Astuti et al. 2001b) and in two of five informative phaeochromocytomas with 11p15.5 allele loss there was no evidence of allele loss close to SDHD.

The 11p15.5-imprinted gene cluster has been implicated in Beckwith–Wiedemann syndrome (BWS) and sporadic embryonal and adult tumours. BWS is characterized by pre- and postnatal overgrowth, developmental anomalies and susceptibility to embryonal tumours, particularly Wilms’ tumour (Maher & Reik 2000). Interestingly, an association of phaeochromocytoma with BWS and isolated hemihyper trophy has been reported on several occasions (Schnakenburg et al. 1976, Bemurat et al. 2002, van den Akker et al. 2002). An association between BWS and adrenocortical tumours is recognized and 11p15.5 abnormalities (e.g. loss of the maternal allele, paternal allele duplication and insulin-like growth factor 2 (IGF2) overexpression) are a common feature of sporadic adrenocortical tumours (Gicquel et al. 1997, Steenman et al. 2000, Gicquel et al. 2001).

The 11p15.5-imprinted gene cluster contains multiple candidate growth regulators, but three genes, the paternally expressed growth promoter IGF2 and the maternally expressed candidate tumour-suppressor genes CDKN1C and H19, have been most consistently implicated in the pathogenesis of BWS and susceptibility to neoplasia. Studies in children with BWS and transgenic mice have led to the identification of two distinct imprinting control elements (IC1 and IC2) marked by differentially methylated regions (H19 CTCF box and KvDMR1 respectively). Epimutation (maternal-allele methylation) at IC1 leads to biallelic IGF2 expression and H19 silencing (Reik et al. 1994). Epimutation (maternal-allele loss of methylation) at KvDMR1 is associated with reduced CDKN1C expression, and in some cases biallelic IGF2 expression (Lee et al. 1999, Smilinich et al. 1999, Diaz-Meyer et al. 2003). Maternal 11p15.5 allele loss will be associated with loss of CDKN1C and H19 expression and expression of both of these genes was reported to be decreased in phaeochromocytomas when compared with normal adrenal (Liu et al. 1997). In addition we detected loss of maternal-allele KvDMR1 methylation in a phaeochromocytoma without 11p15.5 allele loss. This suggests that in this case an epigenetic change (an ‘epimutation’) at KvDMR1 might promote tumourigenesis by downregulation of CDKN1C (and possibly increased IGF2 expression). KvDMR1 demethylation has also been observed in a variety of cancers, including liver, breast, cervical and gastric, but somatic CDKN1C mutations have not been reported (Seelfo et al. 2002).

Phaeochromocytomas arise from chromaffin cells derived from primitive cells of the neural crest that migrate into the paravertebral sympathetic ganglia and from there into the adrenal primordium, whereas
neuroblastomas are composed of histologically primitive neuronal cells. Gene-expression patterns in neuroblastoma and phaeochromocytoma reflect the differing origins of the tumours (particularly for undifferentiated neuroblastomas; Hoehner et al. 1998), but there does appear to be some overlap in the molecular mechanisms of tumourigenesis. Thus we found similar frequencies of methylation of FLIP, TSP1, DcR1, DcR2, DR4, DR5, CASP8 and HIC1 in phaeochromocytoma and neuroblastoma and these findings suggest that tumour-suppressor genes inactivated in neuroblastoma can reasonably be considered as candidate phaeochromocytoma-susceptibility genes. Thus further comparisons of patterns of epigenetic inactivation of tumour suppressor genes will be of interest. Nevertheless phaeochromocytomas are genetically heterogeneous, and we found differences between the frequency of HIC1 and CASP8 methylation between VHL-associated and sporadic phaeochromocytomas. Further definition of the mechanisms of tumourigenesis in VHL and sporadic phaeochromocytomas will provide insights into the role of how different genetic and epigenetic events interact in tumour development.

Acknowledgements

We thank the British Heart Foundation, Cancer Research UK, the University of Birmingham, Deutsche Forschungsgemeinschaft Grant NE 571/5-2 and the Deutsche Krebshilfe Grant 70-33131-Ne 1 for financial support.

References

A
dt
i
D
, D
ouglas
F
, L
ennard
T
W
, Aligianis
I
A
, W
oodward
E
R
, E
vans
D
G
, E
ng
C
, L
atif
F
& M
aher
E
R
 2001a
A
dt
i
D
, L
atif
F
, Da
dol
L
A
, D
ahia
P
L
, D
ouglas
F
, G
eorge
E
, S
koldberg
F
, H
usebye
E
S
, E
ng
C
& M
aher
E
R
 2001b
A
dt
i
D
, A
gathanggelou
A
, H
onorio
S
, Da
dol
L
A
, M
artinson
T
, K
ogner
P
, C
ummins
C
, N
eumann
H
P
, V
outilainen
R
, D
ahia
P
et
al
 2001c
A
dt
i
D
, H
tart-Holden
N
, L
atif
F
, La
dl
oo
F
, B
lack
G
C
, L
im
C
, M
oran
A
, G
rossman
A
B
, H
odgson
S
V
, F
remont
A
et
al
2003
B
aysai
B
E
, F
errell
R
E
, W
illet
-Brozick
J
E
, L
awrence
E
C
, M
yssiorek
D
, B
osch
A
, v
an
der
M
ey
A
, T
aschner
P
E
, R
ubinstein
W
S
, M
yers
E
et
al
2000
B
emurat
L
, G
osse
P
, B
allanger
P
, T
auzin-Fin
P
, B
arat
P
, L
acombe
D
, L
emetary
P
& C
lementy
J
2002
B
ender
B
U
, G
utsche
M
, G
lasker
S
, M
uller
B
, K
irste
G
, E
ng
C
& N
eumann
H
P
 2000
C
han
A
O
, K
im
S
G
, B
deer
A
, I
ssa
J
P
, H
amilton
S
R
& R
ashid
A
 2003
C
hen
W
Y
, Z
eng
X
, C
arter
M
G
, M
orrell
C
N
, C
hiu
Yen
R
W
, E
steller
M
, W
atkins
D
N
, H
erman
J
G
, M
ankowski
J
L
 & B
aylin
S
B
 2003
C
lifford
S
C
, C
ockman
M
E
, S
mallwood
A
C
, M
ole
D
R
, W
oodward
E
R
, M
axwell
P
M
, R
atcliffe
P
J
 & M
aher
E
R
 2001
D
e
Pret
er
K
, V
andesompele
J
, H
oebecke
J
, V
andenbroecke
C
, S
met
J
, N
uysts
A
, L
aureys
G
, C
ombaret
V
, V
an
Roy
N
, R
oels
F
et
al
2004
No evidence for involvement of SDHD in neuroblastoma pathogenesis. BMC Cancer 4:55.
D
iaz-Meyer
N
, D
ay
C
, K
hatod
K
, M
aher
E
R
, C
ooper
W
, R
eik
W
, J
unien
W
, G
raham
G
, A
lgar
E
, D
er
K
alousitian
V
M
 & H
iggins
M
J
 2003
Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KDMR1 in Beckwith-Wiedemann syndrome. Journal of Medical Genetics 40:797–801.
D
ong
S
M
, K
im
H
, R
ha
S
 & S
idransky
D
 2001
E
gger
T
, Gro
tzer
M
A
, Z
uzak
T
J
, V
iewrodt
B
R
, H
o
R
, I
kekagi
N
 & B
roeder
G
M
 2001
E
jeskar
K
, A
buratani
H
, A
brahamson
J
, K
ogner
P
 & M
artinson
T
 1998


Shivapurkar N, Toyooka S, Toyooka KO, Reddy J, Miyajima K, Suzuki M, Shimematsu H, Takahashi T, Parikh G, Pass HI et al. 2004 Aberrant methylation of Trail decoy receptor genes is frequent in multiple...


