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 pheochromocytomas

C D E Margetts¹, D Astuti¹, D C Gentle^{1,2}, W N Cooper¹, A Cascon³, D Catchpoole⁴, M Robledo³, H P H Neumann⁵, F Latif^{1,2} and E R Maher^{1,2}

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

Pheochromocytoma 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 pheochromocytoma 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 pheochromocytoma 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 pheochromocytomas. We identified frequent aberrant methylation of *HIC1* (82%) and *CASP8* (31%) in pheochromocytoma, but both genes were significantly more methylated in VHL pheochromocytomas than in sporadic cases. Of four tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors analysed, DR4 was most commonly methylated (41%; compared with DcR2 (26%), DcR1 (23%) and DR5 (10%)). Gene methylation patterns in pheochromocytoma and neuroblastoma did not differ significantly suggesting overlapping mechanisms of tumourigenesis. We also investigated the role of 11p15.5-imprinted genes in pheochromocytoma. We found that in 10 sporadic and VHL pheochromocytomas 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 pheochromocytomas.

Endocrine-Related Cancer (2005) 12 161–172

Introduction

Pheochromocytomas 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 pheochromocytoma and cardiovascular disease is the leading cause of death, but about 10% are malignant. As with many human neoplasms, a subset of pheochromocytomas occur in genetically susceptible individuals.

Thus inherited pheochromocytoma 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 pheochromocytomas (Eng *et al.* 1995, Hofstra *et al.* 1996, Astuti *et al.* 2001a, 2001b, 2003). Molecular studies of sporadic pheochromocytomas have demonstrated frequent allele losses on chromosomes 1p, 3p, 11 and 22, but there is evidence that the tumour-development pathways may differ in VHL-related and sporadic pheochromocytomas. Thus Bender *et al.* (2000) and Lui *et al.* (2002) found that when comparing VHL and sporadic pheochromocytomas, 3p and 11 allele loss was more common and 1p and 22 allele loss less frequent in VHL-related tumours.

Neuroblastomas and pheochromocytomas are the commonest neural-crest-derived tumours in children and adults respectively. Although familial neuroblastoma is rare and major susceptibility genes have not yet been isolated, many investigators have attempted to define the somatic genetic and epigenetic alterations associated with neuroblastoma development. Common genetic events include N-Myc amplification and loss of heterozygosity (LOH) at 1p36, 3p, 11q23 and 14q23-qter (Ejeskar *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 pheochromocytomas so it appears that there may be an overlap between the genetic and epigenetic events implicated in neuroblastoma and pheochromocytoma. To further investigate the mechanisms of tumourigenesis in sporadic and familial pheochromocytoma we have investigated the epigenetic status of *FLIP*, *TSP1*, *DcR1*, *DcR2*, *DR4*, *DR5*, *CASP8* and *HIC1* genes in VHL and sporadic pheochromocytomas. In addition in view of the observation that preferential maternal chromosome 11 allele loss in pheochromocytomas 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 pheochromocytomas.

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 pheochromocytomas and 16 sporadic pheochromocytomas). In the 11p15.5 LOH study, a total of 48 samples were analysed (40 sporadic and eight VHL-associated pheochromocytomas). 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 (0.5–1.0 µg) 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 30 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 2 μ M 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. 1 μ g RNA was reverse transcribed using Reverse Transcription Systems and oligo dT primers (Promega) according to the manufacturer's protocols. 1 μ l 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'-AAGGTGAAGGTCTGGAGTCAACG-3' and 5'-CAGCCTTCTCCATGGTGGTGAA-3', resulting in a 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'-6-FamCTGGGCTCTGGGGT-GATTCC-3' (forward) and 5'-CCGAGTGCAGGT-CACAGGGA-3' (reverse). PCR products (121 bp) were then run in an ABI 3730 DNA sequencer with LIZ-500 size standards and analysed with Genemapper v.3 software (Applied Biosystems). Chromosome 11q23.3 allele loss was assessed at D11S1998 using primers 5'-6-FamAGCCATCAACTAGCTTTCCC-3' (forward) and 5'-GGGAGGCACCAACAGATG-3' (reverse).

To determine whether 11p15.5 allele loss was likely to involve the maternal or paternal chromosome, we assessed methylation status at the H19 differentially methylated region (DMR) and KvDMR1. These are

CpG islands that are differentially methylated on the two parental chromosomes. Thus KvDMR1 is methylated on the maternal allele and the H19 DMR is methylated on the paternal allele. In order to assess methylation status, bisulphite-modified DNA was amplified for KvDMR1 using primers 5'-6-FamGTTA-TTTTATATTTAGTTAGTGTGTTTATG-3' (forward) and 5'-TCTTACTAAAAAAGTCCCTAAAAATC-3' (reverse), resulting in a 301 bp fragment, and H19 was amplified using primers 5'-9-TetGTAGGG-TTTTGGTAGGTATAGAG-3' (forward) and 5'-CTTAAATAACCCRAAACRTTCCAC-3' (reverse), resulting in 213 bp PCR products. Amplified products were then digested with BstUI 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 M is 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 phaeochromocytomas and neuroblastomas

We used MSP analysis to investigate the methylation status of eight genes in 39 phaeochromocytomas (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 ($\pm$ s.d.) MI values (number of genes methylated/number of genes tested) were 0.35 ± 0.15 for VHL phaeochromocytomas, 0.23 ± 0.15 for sporadic

Figure 1 MSP of *FLIP*, *TSP1*, *HIC-1*, *CASP8*, *DcR1*, *DcR2*, *DR4* and *DR5* in neuroblastoma (NB) and pheochromocytoma (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.

pheochromocytomas 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 pheochromocytomas ($P>0.2$). However, the frequency of methylation of *HIC1* and *CASP8* was significantly higher in VHL pheochromocytomas than in sporadic pheochromocytomas (100

versus 56%, $P=0.001$; 48 versus 6%, $P=0.006$). The frequency of *HIC1* methylation in VHL pheochromocytoma was higher than in neuroblastoma (100 versus 80%, $P=0.039$), and the frequency of *CASP8* methylation in sporadic pheochromocytoma 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

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

Gene	Proportion of tumours methylated (%)			
	Pheochromocytoma			Neuroblastoma tumours
	All	VHL	Sporadic	
<i>TSP1</i>	23% (9/39)	22% (5/23)	25% (4/16)	30% (6/20)
<i>HIC1</i>	82% (32/39)	100% (23/23)**	56% (9/16)**	80% (16/20)
<i>FLIP</i>	8% (3/39)	13% (3/23)	0% (0/16)	5% (1/20)
<i>CASP8</i>	32% (12/38)	48% (11/23)*	6% (1/15)*	40% (8/20)
<i>DcR1</i>	24% (9/38)	17% (4/23)	33% (5/15)	30% (6/20)
<i>DcR2</i>	26% (10/39)	35% (8/23)	13% (2/16)	40% (8/20)
<i>DR4</i>	41% (16/39)	39% (9/23)	44% (7/16)	35% (7/20)
<i>DR5</i>	10% (4/39)	9% (2/23)	13% (2/16)	0% (0/20)

** $P=0.001$.

* $P=0.023$.

Table 2 Individual tumour-methylation patterns

Tumour type and number	Gene								MI
	<i>FLIP</i>	<i>TSP1</i>	<i>DcR1</i>	<i>DcR2</i>	<i>DR4</i>	<i>DR5</i>	<i>CASP8</i>	<i>HIC1</i>	
PV01	U	U	U	M	U	U	M	M	0.375
PV02	U	U	U	U	U	M	M	M	0.375
PV03	U	U	U	M	M	U	U	M	0.375
PV04	U	U	M	M	U	U	M	M	0.5
PV05	U	U	U	U	U	U	U	M	0.125
PV06	U	M	U	M	U	U	M	M	0.5
PV07	U	U	U	M	U	U	M	M	0.375
PV08	U	U	U	U	M	U	U	M	0.25
PV09	U	U	U	U	M	U	U	M	0.25
PV10	U	M	U	U	M	U	M	M	0.5
PV11	U	U	U	U	M	U	U	M	0.25
PV12	U	U	U	U	M	U	U	M	0.25
PV13	U	M	U	M	U	M	M	M	0.625
PV14	M	U	U	U	U	U	U	M	0.25
PV15	U	U	U	U	M	U	U	M	0.25
PV16	U	U	U	U	U	U	U	M	0.125
PV17	M	U	U	U	M	U	M	M	0.5
PV18	M	M	U	M	U	U	M	M	0.625
PV19	U	U	U	M	U	U	U	M	0.25
PV20	U	M	M	U	M	U	U	M	0.5
PV21	U	U	M	U	U	U	M	M	0.375
PV22	U	U	M	U	U	U	M	M	0.375
PV23	U	U	M	U	U	U	M	M	0.125
PS01	U	U	U	U	M	U	U	M	0.25
PS02	U	U	M	U	M	U	M	M	0.375
PS03	U	M	M	M	M	U	M	M	0.5
PS04	U	M	U	M	M	U	U	M	0.125
PS05	U	U	U	U	U	U	U	M	0.125
PS06	U	M	U	U	U	U	U	M	0.25
PS07	U	M	M	U	M	M	U	M	0.5
PS08	U	U	U	M	U	U	U	U	0.125
PS09	U	U	U	U	M	U	U	U	0.125
PS10	U	U	U	U	U	U	U	U	0
PS11	U	U	M	U	M	U	U	M	0.375
PS12	U	U	M	U	M	M	U	M	0.25
PS13	U	U	U	U	M	M	U	M	0.125
PS14	U	U	M	U	M	U	U	U	0.125
PS15	U	M	M	U	U	U	U	U	0.125
PS16	U	M	M	U	M	U	U	M	0.375
NB01	U	U	U	U	U	U	U	M	0.125
NB02	U	U	M	M	U	U	M	M	0.5
NB03	U	U	U	U	U	U	U	U	0
NB04	U	U	U	U	U	U	U	M	0.125
NB05	U	U	U	M	U	U	U	M	0.25
NB06	U	U	U	U	U	U	U	U	0
NB07	U	U	M	M	M	U	U	M	0.5
NB08	U	U	U	M	U	U	U	M	0.25
NB09	U	U	U	U	U	U	U	U	0
NB10	U	U	U	U	U	U	U	U	0
NB11	U	U	M	M	M	U	M	M	0.625
NB12	U	M	U	U	M	U	U	M	0.375
NB13	M	U	U	U	U	U	M	M	0.375
NB14	U	M	M	U	U	U	M	M	0.5
NB15	U	M	U	U	M	U	M	M	0.5
NB16	U	M	U	M	M	U	M	M	0.625
NB17	U	M	U	U	M	U	U	M	0.375
NB18	U	U	M	M	U	U	M	M	0.5
NB19	U	M	U	U	M	U	M	M	0.5
NB20	U	U	M	M	U	U	U	M	0.375

NB, neuroblastoma; PS, sporadic pheochromocytoma; PV, VHL pheochromocytoma; U, unmethylated; M, methylated.

detected in tumours with *HIC1* methylation. A significant association ($P < 0.05$) was still detected after excluding VHL pheochromocytomas 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 ($TSP1 = 3/8$, $DcR1 = 6/8$, $DcR2 = 8/8$ and $DR4 = 2/8$). We then determined the effect of treatment with 5-aza-dC on transcription of four methylated genes (*DcR1*, *DcR2*, *DR4* 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 pheochromocytomas (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 pheochromocytomas 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 pheochromocytomas without 11p15.5 LOH and 10 tumours with 11p15.5 LOH. In one of the 15 pheochromocytomas 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 pheochromocytomas without allele loss, mean $\pm$ s.d. KvDMR1 MI was 0.217 ± 0.12 and mean H19 DMR MI was 0.343 ± 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 pheochromocytomas with 11p15.5 allele loss (mean $\pm$ s.d.): KvDMR1 MI = 0.096 ± 0.04 and H19 DMR MI = 0.478 ± 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 pheochromocytomas ($n = 7$) 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.

Figure 2 Expression analysis of *DcR1*, *DcR2*, *DR4* and *TSP1* by reverse transcriptase PCR in neuroblastoma cell lines without (–) and with (+) 5-aza-dC (5 AZA) treatment. GAPDH was used as a positive control. PCR product sizes were: *DcR1*, 242 bp; *DcR2*, 243 bp; *DR4*, 222 bp; *TSP1*, 159 bp; GAPDH, 319 bp.

Discussion

Pheochromocytoma and neuroblastoma are both derived from the neural crest, yet pheochromocytoma-susceptibility genes (e.g. *VHL*, *RET*, *NFI*, *SDHB* and *SDHD*) have not been implicated in familial neuroblastoma and *PHOX2B* mutations have been associated with familial neuroblastoma but not pheochromocytoma (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* and *HIC1*). Previously we had demonstrated promoter methylation of the *RASSF1A* tumour-suppressor gene in 22% of sporadic pheochromocytomas and 55% of

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

Tumour ID	11p15.5 status	11q23.3 status	H19 DMR MI	KvDMR1 MI	H19/KvDMR1 MI ratio
PS21	LOH	NI	0.67	0	>10
PS14	RET		0.35	0	>10
PS25	LOH	RET	0.54	0.07	7.7
PS07	LOH	NI	0.6	0.09	6.7
PV16	LOH	LOH	0.46	0.08	5.8
PV07	LOH	NI	0.4	0.08	5
PS30	LOH	NI	0.42	0.11	3.8
PV23	LOH	NI	0.37	0.1	3.7
PS29	LOH	LOH	0.48	0.13	3.7
PS18	LOH	LOH	0.39	0.15	3.3
PS24	LOH	RET	0.45	0.15	3
PS32	RET		0.41	0.15	2.7
PS05	RET		0.33	0.12	2.7
PV22	RET		0.36	0.15	2.4
PS27	RET		0.31	0.13	2.4
PS19	RET		0.26	0.12	2.2
PS34	RET		0.28	0.14	2
PS41	RET		0.37	0.2	1.9
PV15	RET		0.33	0.18	1.8
PV19	RET		0.29	0.23	1.3
PS06	RET		0.45	0.37	1.2
PS33	RET		0.45	0.41	1.1
PS12	RET		0.32	0.32	1.0
PS40	RET		0.31	0.34	0.9
PS17	RET		0.33	0.39	0.8

NI, non informative.

neuroblastomas (Astuti *et al.* 2001c), suggesting that analysis of genes implicated in neuroblastoma tumorigenesis might provide insights into the pathogenesis of pheochromocytoma, but *HIC1*, *FLIP*, *TSP1*, *DeR1*, *DeR2*, *DR4*, *DR5* and *CASP8* had not been analysed previously in pheochromocytomas.

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 pheochromocytomas, so that *HIC1* methylation is not restricted to malignant tumours. Previously we and others demonstrated frequent *CASP8* methylation in neuroblastoma (Teitz *et al.* 2000, Astuti *et al.* 2001c), and we have now also demonstrated that *CASP8* methylation occurs often in pheochromocytoma. Intriguingly we found that (i) *HIC1* and *CASP8* methylation were significantly associated in neuroblastomas and pheochromocytomas (and *CASP8* methylation was only detected

in tumours with *HIC1* methylation) and (ii) *HIC1* and *CASP8* methylation were both significantly more common in VHL-associated than in sporadic pheochromocytomas. These latter observations provide further evidence for differing mechanisms of tumorigenesis in VHL and sporadic pheochromocytomas. Previously chromosome 11 and 3p allele loss was associated preferentially with VHL pheochromocytomas whereas 1p allele loss was significantly more common in sporadic pheochromocytomas (Bender *et al.* 2000, Lui *et al.* 2002). The differential methylation of *HIC1* and *CASP8* in VHL pheochromocytoma 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 pheochromocytomas are vascularized tumours, although the relevance of HIF dysregulation for the development of pheochromocytoma in VHL disease is unclear

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.

(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 carcinoid 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 phaeochromocytoma. 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

TRAIL apoptosis and methylation of at least one or more of these genes was detected in 67% of pheochromocytomas 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 κ B (NF- κ B) pathway through *DR4* and *DR5*. On the other hand, *DcR1* and *DcR2* are thought to inhibit TRAIL-induced NF- κ B 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 pheochromocytoma 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 pheochromocytoma (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 pheochromocytomas 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 pheochromocytomas with germline *SDHD* mutations, but also occurs in VHL and sporadic pheochromocytomas. 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 tumorigenesis. Somatic *SDHD* mutations have not been reported in sporadic pheochromocytomas (Astuti *et al.* 2001b) and in two of five informative pheochromocytomas 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 pheochromocytoma with BWS and isolated hemihypertrophy 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, Smilnich *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 pheochromocytomas when compared with normal adrenal (Liu *et al.* 1997). In addition we detected loss of maternal-allele KvDMR1 methylation in a pheochromocytoma without 11p15.5 allele loss. This suggests that in this case an epigenetic change (an 'epimutation') at KvDMR1 might promote tumorigenesis 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 (Scelfo *et al.* 2002).

Pheochromocytomas 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 pheochromocytoma 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 pheochromocytoma and neuroblastoma and these findings suggest that tumour-suppressor genes inactivated in neuroblastoma can reasonably be considered as candidate pheochromocytoma-susceptibility genes. Thus further comparisons of patterns of epigenetic inactivation of tumour suppressor genes will be of interest. Nevertheless pheochromocytomas are genetically heterogeneous, and we found differences between the frequency of *HIC1* and *CASP8* methylation between VHL-associated and sporadic pheochromocytomas. Further definition of the mechanisms of tumourigenesis in VHL and sporadic pheochromocytomas 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

- Astuti D, Douglas F, Lennard TW, Aligianis IA, Woodward ER, Evans DG, Eng C, Latif F & Maher ER 2001a Germline SDHD mutation in familial pheochromocytoma. *Lancet* **357** 1181–1182.
- Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, Skoldberg F, Husebye ES, Eng C & Maher ER 2001b Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *American Journal of Human Genetics* **69** 49–54.
- Astuti D, Agathangelou A, Honorio S, Dallol A, Martinsson T, Kogner P, Cummins C, Neumann HP, Voutilainen R, Dahia P *et al.* 2001c RASSF1A promoter region CpG island hypermethylation in pheochromocytomas and neuroblastoma tumours. *Oncogene* **20** 7573–7577.
- Astuti D, Hart-Holden N, Latif F, Laloo F, Black GC, Lim C, Moran A, Grossman AB, Hodgson SV, Freemont A *et al.* 2003 Genetic analysis of mitochondrial complex II subunits SDHD, SDHB and SDHC in paraganglioma and pheochromocytoma susceptibility. *Journal of Clinical Endocrinology* **59** 728–733.
- Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinstein WS, Myers EN *et al.* 2000 Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* **287** 848–851.
- Bemurat L, Gosse P, Ballanger P, Tauzin-Fin P, Barat P, Lacombe D, Lemetayer P & Clementy J 2002 Successful laparoscopic operation of bilateral pheochromocytoma in a patient with Beckwith-Wiedemann syndrome. *Journal of Human Hypertension* **16** 281–284.
- Bender BU, Gutsche M, Glasker S, Muller B, Kirste G, Eng C & Neumann HP 2000 Differential Genetic Alterations in von-Hippel-Lindau Syndrome-associated and Sporadic Pheochromocytomas. *Journal of Clinical Endocrinology and Metabolism* **85** 4568–4574.
- Chan AO, Kim SG, Bedeir A, Issa JP, Hamilton SR & Rashid A 2003 CpG island methylation in carcinoid and pancreatic endocrine tumors. *Oncogene* **22** 924–934.
- Chen WY, Zeng X, Carter MG, Morrell CN, Chiu Yen RW, Esteller M, Watkins DN, Herman JG, Mankowski JL & Baylin SB 2003 Heterozygous disruption of Hic1 predisposes mice to a gender-dependent spectrum of malignant tumors. *Nature Genetics* **33** 197–202.
- Clifford SC, Cockman ME, Smallwood AC, Mole DR, Woodward ER, Maxwell PM, Ratcliffe PJ & Maher ER 2001 Contrasting effects on HIF-1 alpha regulation by disease-causing PVHL mutations correlate with patterns of tumourigenesis in von Hippel-Lindau disease. *Human Molecular Genetics* **10** 1029–1038.
- De Preter K, Vandesompele J, Hoebeek J, Vandenbroecke C, Smet J, Nuyts A, Laureys G, Combaret V, Van Roy N, Roels F *et al.* 2004 No evidence for involvement of SDHD in neuroblastoma pathogenesis. *BMC Cancer* **4** 55.
- Diaz-Meyer N, Day C, Khatod K, Maher ER, Cooper W, Reik W, Junien W, Graham G, Algar E, Der Kaloustian VM & Higgins MJ 2003 Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KVDMR1 in Beckwith-Wiedemann syndrome. *Journal of Medical Genetics* **40** 797–801.
- Dong SM, Kim H, Rha S & Sidransky D 2001 Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. *Clinical Cancer Research* **7** 1982–1986.
- Eggert A, Grotzer MA, Zuzak TJ, Wiewrodt BR, Ho R, Ikegaki N & Brodeur GM 2001 Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-9 expression. *Cancer Research* **61** 1314–1319.
- Ejeskar K, Aburatani H, Abrahamsson J, Kogner P & Martinsson T 1998 Loss of heterozygosity of 3p markers in neuroblastoma tumours implicate a tumour-suppressor locus distal to the FHIT gene. *British Journal of Cancer* **77** 1787–1791.

- Eng C, Crossey PA, Mulligan LM, Healey CS, Houghton C, Prowse A, Chew SL, Dahia PL, O'Riordan JL & Toledo SP 1995 Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic pheochromocytomas. *Journal of Medical Genetics* **32** 934–937.
- Gicquel C, Raffin-Sanson ML, Gaston V, Bertagna X, Plouin PF, Schlumberger M, Louvel A, Luton JP & Le Bouc Y 1997 Structural and functional abnormalities at 11p15 are associated with the malignant phenotype in sporadic adrenocortical tumors: study on a series of 82 tumors. *Journal of Clinical Endocrinology and Metabolism* **82** 2559–2565.
- Gicquel C, Bertagna X, Gaston V, Coste J, Louvel A, Baudin E, Bertherat J, Chapuis Y, Duclos JM, Schlumberger M *et al.* 2001 Molecular markers and long-term recurrences in a large cohort of patients with sporadic adrenocortical tumors. *Cancer Research* **61** 6762–6767.
- Hensen EF, Jordano ES, Van Minderhout IJ, Hogendoorn PC, Taschner PE, Van Der Mey AG, Devilee P & Cornelisse CJ 2004 Somatic loss of maternal chromosome 11 causes parent-of-origin-dependent inheritance in SDHD-linked paraganglioma and pheochromocytoma families. *Oncogene* **23** 4076–4083.
- Herman JG, Graff JR, Myohanen S, Nelkin BD & Baylin SB 1996 Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *PNAS* **93** 9821–9826.
- Hoehner JC, Hedborg F, Eriksson L, Sandstedt B, Grimelius L, Olsen L & Pahlman S 1998 Developmental gene expression of sympathetic nervous system tumors reflects their histogenesis. *Laboratory Investigation* **78** 29–45.
- Hoffman MA, Ohh M, Yang H, Klc JM, Ivan M & Kaelin WG Jr 2001 von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Human Molecular Genetics* **10** 1019–1027.
- Hofstra RM, Stelwagen T, Stulp RP, de Jong D, Hulsbeek M, Kamsteeg EJ, van den Berg A, Landsvater RM, Vermey A, Molenaar WM *et al.* 1996 Extensive mutation scanning of RET in sporadic medullary thyroid carcinoma and of RET and VHL in sporadic pheochromocytoma reveals involvement of these genes in only a minority of cases. *Journal of Clinical Endocrinology and Metabolism* **81** 2881–2884.
- Lee MP, DeBaun MR, Mitsuya K, Galonek HL, Brandenburg S, Oshimura M & Feinberg AP 1999 Loss of imprinting of a paternally expressed transcript, with antisense orientation to KCNQ1, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. *PNAS* **96** 5203–5208.
- Liu J, Kahri AI, Heikkila P & Voutilainen R 1997 Ribonucleic acid expression of the clustered imprinted genes, p57KIP2, insulin-like growth factor II, and H19, in adrenal tumors and cultured adrenal cells. *Journal of Clinical Endocrinology and Metabolism* **82** 1766–1771.
- Lui WO, Chen J, Glasker S, Bender BU, Madura C, Khoo SK, Kort E, Larsson C, Neumann HP & Teh BT 2002 Selective loss of chromosome 11 in pheochromocytomas associated with the VHL syndrome. *Oncogene* **21** 1117–1122.
- Maher ER & Reik W 2000 Beckwith-Wiedemann syndrome imprinting in clusters revisited. *Journal of Clinical Investigation* **105** 247–252.
- Maher ER & Eng C 2002 The pressure rises: update on the genetics of pheochromocytoma. *Human Molecular Genetics* **11** 2347–2354.
- Maris JM & Matthay KK 1999 Molecular biology of neuroblastoma. *Journal of Clinical Oncology* **17** 2264–2279.
- Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER & Ratcliffe PJ 1999 The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399** 203–204.
- Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Althoefer C, Zerres K *et al.* 2002 Germ-line Mutations in nonsyndromic Pheochromocytoma. *New England Journal of Medicine* **346** 1459–1466.
- Oue N, Matsumura S, Nakayama H, Kitadai Y, Taniyama K, Matsusaki K & Yasui W 2003 Reduced expression of the TSP1 gene and its association with promoter hypermethylation in gastric carcinoma. *Oncology* **64** 423–429.
- Rathi A, Virmani AK, Harada K, Timmons CF, Miyajima K, Hay RJ, Mastrangelo D, Maitra A, Tomlinson GE & Gazdar AF 2003 Aberrant methylation of the HIC1 promoter is a frequent event in specific pediatric neoplasms. *Clinical Cancer Research* **9** 3674–3678.
- Reik W, Brown KW, Slatter RE, Sartori P, Elliott M & Maher ER 1994 Allelic methylation of H19 and IGF2 in the Beckwith-Wiedemann syndrome. *Human Molecular Genetics* **3** 1297–1301.
- Scelfo RA, Schwienbacher C, Veronese A, Gramantieri L, Bolondi L, Querzoli P, Nenci I, Calin GA, Angioni A, Barbanti-Brodano G & Negrini M 2002 Loss of methylation at chromosome 11p15.5 is common in human adult tumors. *Oncogene* **21** 2564–2572.
- Schnakenburg KV, Muller M, Dorner K, Harms D & Schwarze EW 1976 Congenital hemihypertrophy and malignant giant pheochromocytoma. A previously undescribed coincidence. *European Journal of Pediatrics* **122** 263–273.
- Shivapurkar N, Toyooka S, Toyooka KO, Reddy J, Miyajima K, Suzuki M, Shigematsu H, Takahashi T, Parikh G, Pass HI *et al.* 2004 Aberrant methylation of trail decoy receptor genes is frequent in multiple

- tumor types. *International Journal of Cancer* **109** 786–792.
- Smilnich NJ, Day CD, Fitzpatrick GV, Caldwell GM, Lossie AC, Cooper PR, Smallwood AC, Joyce JA, Schofield PN, Reik W *et al.* 1999 A maternally methylated CpG island in KCNQ1 is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. *PNAS* **96** 8064–8069.
- Steenman M, Westerveld A & Mannens M 2000 Genetics of Beckwith-Wiedemann syndrome-associated tumors: common genetic pathways. *Genes Chromosomes Cancer* **28** 1–13.
- Teitz T, Wei T, Valentine MB, Vanin EF, Grenet J, Valentine VA, Behm FG, Look AT, Lahti JM & Kidd VJ 2000 Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nature Medicine* **6** 529–535.
- Trochet D, Bourdeaut F, Janoueix-Lerosey I, Deville A, de Pontual L, Schleiermacher G, Coze C, Philip N, Frebourg T, Munnich A *et al.* 2004 Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. *American Journal of Human Genetics* **74** 761–764.
- van den Akker EL, de Krijger RR, de Herder WW & Drop SL 2002 Congenital hemihypertrophy and pheochromocytoma, not a coincidental combination? *European Journal of Pediatrics* **161** 157–160.
- van der Mey AG, Maaswinkel-Mooy PD, Cornelisse CJ, Schmidt PH & van de Kamp JJ 1989 Genomic imprinting in hereditary glomus tumours: evidence for new genetic theory. *Lancet* **2** 1291–1294.
- van Noesel MM, van Bezouw S, Salomons GS, Voute PA, Pieters R, Baylin SB, Herman JG & Versteeg R 2002 Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation. *Cancer Research* **62** 2157–2161.
- van Noesel MM, van Bezouw S, Voute PA, Herman JG, Pieters R & Versteeg R 2003 Clustering of Hypermethylated Genes in Neuroblastoma. *Genes Chromosomes and Cancer* **38** 226–233.
- Yang Q, Liu S, Tian Y, Salwen HR, Chlenski A, Weinstein J & Cohn SL 2003 Methylation-associated silencing of the thrombospondin-1 gene in human neuroblastoma. *Cancer Research* **63** 6299–6310.