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

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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 DcR2 (26%), DcR1 (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.

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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.

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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 (Eng *et al*. 1995, Hofstra *et al*. 1996, Astuti *et al*. 2001*a*, 2001*b*, 2003). Molecular studies of sporadic phaeochromocytomas 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 phaeochromocytomas. Thus Bender *et al*. (2000) and Lui *et al*. (2002) found that when comparing VHL and sporadic phaeochromocytomas, 3p and 11 allele loss was more common and 1p and 22 allele loss less frequent in VHL-related tumours.

Neuroblastomas and phaeochromocytomas 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*. 2001*c*). *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 (0.5–1.0 μg) was denatured at 37 °C for 10 min in 0.3 M NaOH. Unmethylated cytosines were sulphonated by incubation in 3.12 Msodium 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 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 MNaOH 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 H_2O at 2 mg/ml and filter-sterilized. 1×10^6 cells were plated in 75 cm^2 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 μM 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 μg 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′-AAGGTGAAGGTCGGAGTCAACG-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-FamCTGGGCTCTGGGGTGATTCC-3′ (forward) and 5′-CCGAGTGCAGGTCACAGGGA-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-FamGTTATTTTATATTTAGTTAGTGTTTTATG-3′ (forward) and 5′-TCTTACTAAAAAACTCCCTAAAAATC-3′ (reverse), resulting in a 301 bp fragment, and H19 was amplified using primers 5′-9-TetGTAGGGTTTTTGGTAGGTATAGAG-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 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 phaechromocytomas 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 phaeochromoctromas, 0.23 ± 0.15 for sporadic

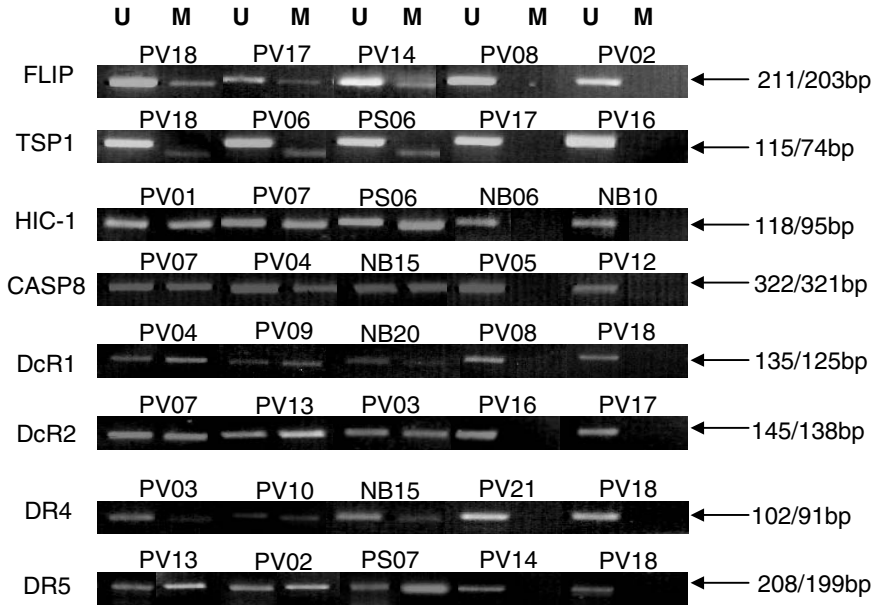

Figure 1 MSP of *FLIP*, *TSP1*, *HIC-1*, *CASP8*, *DcR1*, *DcR2*, *DR4* and *DR5* in neuroblastoma (NB) and phaeochromocytoma (PV) tumours. Bisulphite-modified DNA was amplified with primers specific for unmethylated (U) and methylated (M) DNA. Sample numbers are indicated above the bands. Sizes of the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

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

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

	Proportion of tumours methylated (%)			
	Phaeochromocytoma			
Gene	All	VHL	Sporadic	Neuroblastoma tumours
TSP1	23% (9/39)	22% (5/23)	25% (4/16)	30% (6/20)
HIC1	82% (32/39)	100% (23/23)**	56% (9/16)**	80% (16/20)
FLIP	8% (3/39)	13% (3/23)	0% (0/16)	5% (1/20)
CASP8	32% (12/38)	48% (11/23)*	6% (1/15)*	40% (8/20)
DcR1	24% (9/38)	17% (4/23)	33% (5/15)	30% (6/20)
DcR2	26% (10/39)	35% (8/23)	13% (2/16)	40% (8/20)
DR4	41% (16/39)	39% (9/23)	44% (7/16)	35% (7/20)
DR5	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
	FLIP	*TSP1*	*DcR1*	*DcR2*	*DR4*	*DR5*	*CASP8*	*HIC1*	
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	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 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 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 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 ± 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 phaeochromocytomas with 11p15.5 allele loss (mean $\pm$ S.D.): KvDMR1 MI $=0.096\pm0.04$ and H19 DMR MI $=0.478\pm0.1$.

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.

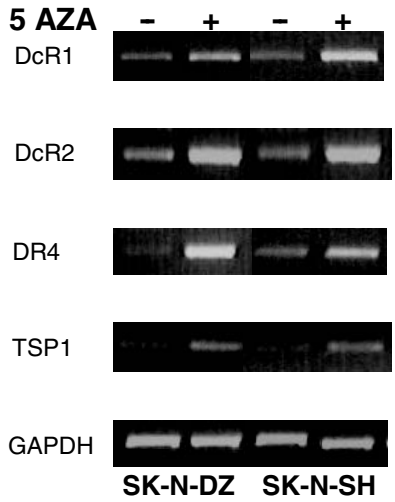

Figure 2 Expression analysis of *DcR1*, *DcR2*, *DR4* and *TSP1* by reverse transcriptase PCR in neuroblastoma cell lines without (–) and with (+) 5-aza-dC (5 AZA) treatment. GAPDH was used as a positive control. PCR product sizes were: DcR1, 242 bp; DcR2, 243 bp; DR4, 222 bp; TSP1, 159 bp; GAPDH, 319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

Discussion

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* and *HIC1*). Previously we had demonstrated promoter methylation of the RASSF1A tumour-suppressor gene in 22% of sporadic phaeochromocytomas and 55% of

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

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*. 2001*c*), 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 we and others demonstrated frequent *CASP8* methylation in neuroblastoma (Teitz *et al*. 2000, Astuti *et al*. 2001*c*), and we have now also demonstrated that *CASP8* methylation occurs often in phaeochromocytoma. Intriguingly we found that (i) *HIC1* and *CASP8* methylation were significantly associated in neuroblastomas and phaeochromocytomas (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 phaeochromocytomas. These latter observations provide further evidence for differing mechanisms of tumourigenesis in VHL and sporadic phaeochromocytomas. 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

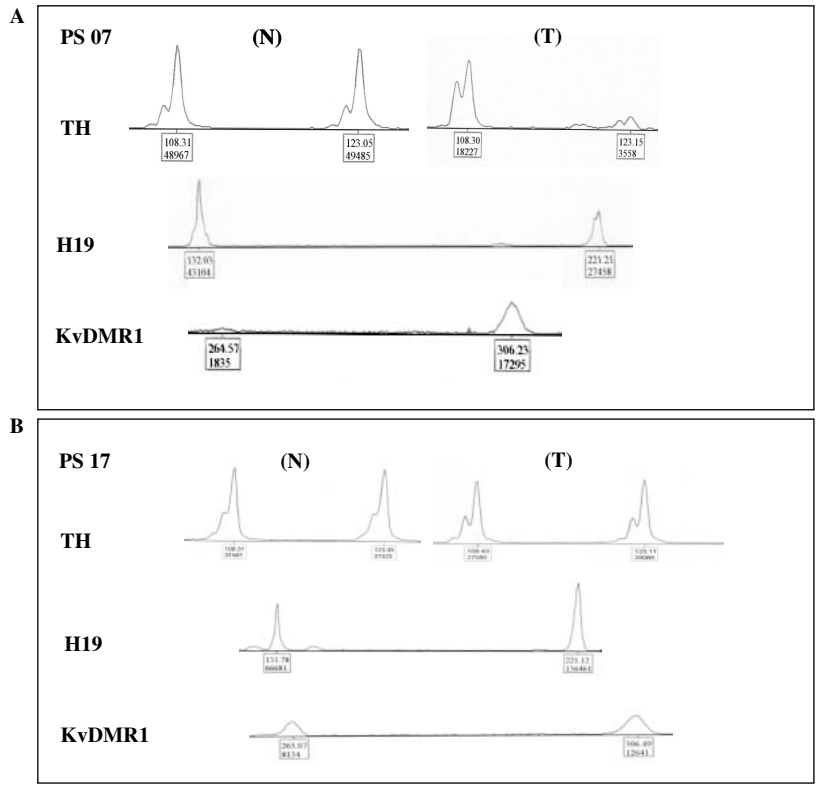

Figure 3 (A) LOH at the TH locus and loss of methylation at KvDMR1 in tumour PS07 and (B) retention of heterozygosity at the TH locus and normal H19 and KvDMR1 methylation in tumour PS17. Boxes: upper label indicates the length of the fragment (H19, 132 bp methylated paternal allele and 221 bp unmethylated 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-apoptosic, 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 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 tumourigenesis. In this context it is interesting to note that TRAIL can activate the anti-apoptotic nuclear factor κB (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*. 2001*a*). 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*. 2001*b*) 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 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 aderenocortical 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 (Scelfo *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.

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