Evaluation of a functional epigenetic approach to identify promoter region methylation in phaeochromocytoma and neuroblastoma

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Abstract

The molecular genetics of inherited phaeochromocytoma have received considerable attention, but the somatic genetic and epigenetic events that characterise tumourigenesis in sporadic phaeochromocytomas are less well defined. Previously, we found considerable overlap between patterns of promoter region tumour suppressor gene (TSG) hypermethylation in two neural crest tumours, neuroblastoma and phaeochromocytoma. In order to identify candidate biomarkers and epigenetically inactivated TSGs in phaeochromocytoma and neuroblastoma, we characterised changes in gene expression in three neuroblastoma cell lines after treatment with the demethylating agent 5-azacytidine. Promoter region methylation status was then determined for 28 genes that demonstrated increased expression after demethylation. Three genes *HSP47*, homeobox A9 (*HOXA9*) and opioid binding protein (*OPCML*) were methylated in >10% of phaeochromocytomas (52, 17 and 12% respectively). Two of the genes, epithelial membrane protein 3 (*EMP3*) and *HSP47*, demonstrated significantly more frequent methylation in neuroblastoma than phaeochromocytoma. These findings extend epigenotype of phaeochromocytoma and identify candidate genes implicated in sporadic phaeochromocytoma tumourigenesis.

Endocrine-Related Cancer (2008) **15** 777–786

Introduction

Neuroblastoma and phaeochromocytoma are the commonest neural crest-derived tumours in children and adults respectively. Most phaeochromocytomas are benign catecholamine-producing tumours arising within the adrenal medulla, but may be extra-adrenal (when they may be designated as paragangliomas) and/or approximately malignant (Chrisoulidou *et al*. 2007, Disick & Palese 2007). Up to one-third of phaeochromocytomas

occur in genetically susceptible individuals (Astuti *et al*. 2001*a*,*b*, Neumann *et al*. 2002, Gimenez-Roqueplo 2006). Inherited predisposition to phaeochromocytoma may be associated with mutations in *NF1*, *RET*, *SDHB*, *SDHC*, *SDHD* or *VHL* genes, but somatic mutations in *VHL*, *RET*, *NF1*, *SDHB* and *SDHD* are rare in sporadic phaeochromocytomas (Eng *et al*. 1995, Hofstra *et al*. 1996, Astuti *et al*. 2001*b*,*c*, 2003). Promoter region hypermethylation and transcriptional silencing is a

frequent cause of tumour suppressor gene (TSG) inactivation in many human cancers. Previously, in order to identify candidate epigenetically inactivated TSGs in phaeochromocytoma, we analysed promoter methylation status in a series of candidate genes known to undergo epigenetic silencing and found similar patterns of promoter hypermethylation in neuroblastoma and phaeochromocytoma. Thus, *TSP1*, *CASP8*, *HIC1*, *DcR1*, *DcR2* and *DR4* and *RASSF1* were methylated frequently in both tumour types (Astuti *et al.* 2001*c*, Margetts *et al.* 2005). In contrast to phaeochromocytoma, familial neuroblastoma is rare. However, the genetic and epigenetic events associated with tumourigenesis in sporadic neuroblastoma are better defined than in phaeochromocytoma and molecular investigations of phaeochromocytoma tumourigenesis have been hampered by the absence of a human phaeochromocytoma cell line.

Based on our previous studies, we hypothesised that the identification of novel genes exhibiting promoter methylation in neuroblastoma would also provide plausible candidate genes for phaeochromocytoma tumourigenesis. In order to expand the number of candidate 'epigenetically silenced TSGs', we investigated a functional epigenomic approach (Alaminos *et al.* 2005) in neuroblastoma cell lines in order to identify candidate epigenetically inactivated TSGs in neuroblastoma and phaeochromocytoma.

Patients and methods

Patients and samples

DNA from a total of 52 tumour samples were analysed (19 NB's, 19 VHL-associated phaeochromocytomas and 14 sporadic phaeochromocytomas). Informed consent and approval from the appropriate Institutional Review Boards were obtained for all samples. DNA was extracted by standard methods. Normal human adult adrenal cDNA and genomic DNA were obtained from AMS Biotechnology (Europe) Ltd (Abingdon, Oxon, UK).

Cell lines

The ten neuroblastoma cell lines used were SK-N-AS, SK-N-F1, SK-N-DZ, SK-N-MC, SK-N-BE, SK-N-SH, NMB and LAN-5 (American Type Culture Collection, Manassas, VA, USA), KELLY and CHP212.

Sodium bisulphate 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 and 1 M hydroquinone (pH 5) at (95 °C (30 s) 50 °C (15 min)) for 20 cycles. The resulting sulphonated DNA was purified using the Wizard DNA clean-up system (Promega), 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 NaOAc (5 μl of 3 M) and ethanol (125 μl of 100%) overnight at −20 °C and resuspended in 50 μl distilled water.

Direct bisulphate DNA sequencing and methylation-specific PCR (MSP)

We determined CpG island methylation status by direct sequencing of bisulphite-modified genomic DNA or MSP.

For direct bisulphite sequencing, PCR products were excised from agarose gels and extracted using the QIAquick Gel Extraction kit (Qiagen), according to the manufacturer's instructions. Products were confirmed by direct sequencing from the forward or reverse PCR primer using ABI PRISM BigDye Terminator v3.1 Cycle sequencing kit (Genpak Ltd, Brighton, Sussex, UK) according to the manufacturer's instructions and run using ABI PRISM 3700 automatic sequencer (See Supplementary Table 1 which can be viewed online at http://erc.endocrinology-journals.org/supplemental/ for primers and PCR conditions).

MSP was performed essentially using previously published primers and conditions (Conway *et al.* 2000, Sellar *et al.* 2003, Yang *et al.* 2004, Alaminos *et al.* 2005, Breault *et al.* 2005, Lind *et al.* 2006). Expected PCR products are as follows: for *HSP47*(a), 134 (methylated)/135 (unmethylated) bp; *HSP47*(b), 109 (methylated)/110 (unmethylated) bp; *EMP3*, 252 (methylated)/255 (unmethylated) bp; opioid binding protein (*OPCML*), 135 (methylated)/135 (unmethylated) bp; *CTNNG*, 95 (methylated)/85 (unmethylated) bp; *SYK*, 242 (methylated)/140 (unmethylated); *TMS1*, 212 (methylated)/209 (unmethylated) bp; Homeobox A9 (*HOXA9*), 127 (methylated)/139 (unmethylated) bp (see Supplementary Table 1 which can be viewed online at http://erc.endocrinology-journals.org/supplemental/).

Reactions were hot-started at 95 °C for 15 min, by using 0.25 μl (5 units/μl) of HotstarTaq DNA polymerase (Qiagen). PCR products were visualised on 2% agarose gels stained with ethidium bromide. Genomic DNA methylated *in vitro* using *SssI* methylase (New England Biolabs, Ipswich, MA, USA) was used as a positive control for MSP and direct bisulphite sequencing.

Treatment of cell lines with 5-aza-2′-deoxycytidine

5-aza-2′-deoxycytidine (5-aza-dC, Sigma) was freshly prepared in ddH$_2$O at 2 mg/ml and filter sterilised. Cells (1×10^6) were plated in 75 cm^2 flask in RPMI 1640 medium supplemented with 10% FCS and left to settle for 24 h (day 0). Cells were treated with 2 μM of 5-aza-dC at days 1 and 4 and harvested at 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.

U133A Plus Affymetrix oligonucleotide array analysis of neuroblastoma cell lines and gene expression analysis

Total RNA was isolated from the 5-aza-dC-treated and untreated neuroblastoma cell lines (SK-N-F1, SK-N-BE and SK-N-DZ) using the RNeasy Mini kit (Qiagen) and subsequently cleaned using the RNeasy mini columns (Qiagen) according to manufacturer's instructions. The quality and integrity of the RNA was verified by checking 28S and 18S rRNA after ethidium bromide staining of total RNA samples on 1% agarose gel electrophoresis. cDNA was performed using the SuperScript Double Stranded cDNA Synthesis kit (Invitrogen). cRNA was synthesised by *in vitro* transcription with biotinylated UTP and CTP. Labelled nucleic acid target quality was assessed by test 2 arrays and hybridised (45 °C for 16 h) to Affymetrix Human U133A plus oligonucleotide arrays. After automated washing and staining, absolute values of expression were calculated and normalised from the scanned array by using Affymetrix Microarray Suite (Version 5.0; Santa Clara, CA, USA). The Affymetrix RNA microrray was performed by Light Laboratories (Dr E Smith), University of Leeds using standard procedures.

Gene expression analysis for individual genes was performed by reverse transcription-PCR (RT-PCR). One microgram of RNA was reverse transcribed using Reverse Transcription Systems and oligo dT primers (Promega) according to the manufacturer's protocols. One microlitre of the cDNA obtained was then used as template for PCR amplification. Primer sequences and conditions are detailed in Supplementary Table 2, which can be viewed online at http://erc.endo-crinology-journals.org/supplemental/. As a control, the GAPDH primers used were: 5′-AAGGTGAA GGTCGGAGTCAACG-3′ and 5′-CAGCCTTCTCCA TGGTGGTGAA-3′, resulting in a PCR product of 319 bp. PCR products were visualised on 2% agarose gels stained with ethidium bromide.

Statistical analysis

Fisher's exact test was used as appropriate. *P* values of <0.05 were taken as statistically significant.

Results

Identification and evaluation of differentially expressed genes after demethylation of three neuroblastoma cell lines

Following treatment with the demethylating agent 5-aza-dC (5 μmol/l) for 5 days to reactivate the epigenetically silenced/downregulated genes, gene expression patterns pre- and post-treatment were compared using Gene Expression Microarrays (Affymetrix HG-U133A) in three neuroblastoma cell lines (SK-N-F1, SK-N-BE and SK-N-DZ). Candidate epigenetically silenced genes were prioritised for possible further investigation if a) they were known to be expressed in normal adrenal tissue (http://genome. ucsc.edu/cgi-bin/hgGateway), b) they were not located on the X chromosome or known to be imprinted, c) there was a CpG island proximal to the transcriptional start site that contained a predicted promoter sequence and d) treatment with 5-aza-dC was associated with a >2-fold upregulation, in at least two cell lines (or one cell line for genes previously implicated in human tumourigenesis).

Evaluation of candidate gene selection criteria for genes known to be epigenetically inactivated in neural crest tumours

Previously, we identified promoter region methylation in neuroblastoma and phaeochromocytoma for *DcR1*, *DcR2*, *DR4*, *CASP8* and *TSP1*. To evaluate our candidate gene selection criteria, we investigated the correlation between promoter methylation status and fold changes in expression for these five genes in the three cell lines. Generally, there was a good correlation e.g. *DcR1*, which is methylated in SK-N-F1 and SK-N-DZ, but not in SK-N-BE was associated with microarray expression changes of 2.77-, 2.47- and 0.47-fold respectively. If the proposed selection criteria had been applied to these five genes then four (*DcR1*, *DcR2*, *DR4* and *CASP8*) would have been selected for further analysis. We then applied our criteria and selected 27 candidate genes for further investigation (Table 1). Prior to performing 5′ CpG island promoter methylation, we evaluated the validity of the gene expression microarray analysis results for 10 genes (*K19*, Stannin (*SNN*), *RASD1*, actin-related protein 2/3 complex, sub unit 4 (*ARPC4*), protein tyrosine kinase 2 beta (*PTK2B*), transcription factor

Table 1 Data from microarray analysis showing candidate genes with greatest fold changes in expression after treatment with 5-aza-dC. Genes in bold were already known to have a role in the pathogenesis of neuroblastomas

Affy ID	Gene	Gene name	SK-N-F1	SK-N-BE	SK-N-DZ
201650_at	KRT19	Keratin 19	1.95	3.73	63.71
218033_s_at	SNN	Stannin	3.76	1.00	50.50
206215_at	**OPCML**	**Opioid binding protein**	**20.52**	**3.52**	**0.26**
223467_at	RASD1	Activator of G protein signalling (AGS1)	19.64	3.03	0.65
217817_at	ARPC4	Actin-related protein 2/3 complex, sub unit 4	11.99	1.02	3.98
203110_at	PTK2B	Protein tyrosine kinase 2 beta	2.73	10.95	2.02
205688_at	TFAP4	Transcription factor AP-4	2.45	7.45	4.96
201367_s_at	ZFP36L2	Zinc finger protein 36, C3H type-like 2	2.98	2.96	8.71
201167_x_at	ARHGDIA	ρ GDP dissociation inhibitor (GDI) α	5.51	1.50	3.88
231248_at	CST6	Cystatin E/M	2.73	2.5	5.62
202062_s_at	SEL1L	Suppressor of lin-12-like	2.64	2.97	5.12
219922_s_at	LTBP3	Latent-transforming growth factor β-binding protein 3	0.82	3.60	5.17
209427_at	SMTH	Smoothelin	2.91	2.59	3.88
205346_at	ST3GAL2	ST3 β-galactoside α-2,3-sialyltransferase 2	3.77	1.43	3.71
201015_s_at	**JUP**	**Junction plakoglobin/CTNNG**	**1.44**	**3.08**	**3.50**
209878_s_at	RELA	v-rel reticuloendotheliosis viral oncogene homologue A	1.73	3.32	2.74
209905_at	**HOXA9**	**Homeobox A9**	**0.94**	**2.71**	**4.13**
204911_s_at	TRIM3	Tripartite motif-containing 3	2.07	1.49	4.09
217250_s_at	CHD5	Chromodomain helicase DNA -binding protein 5	0.06	3.36	3.9
208325_s_at	PRKA2R	A kinase (PRKA) anchor protein 13	0.85	3.94	2.30
202588_at	AK1	Adenylate kinase 1	2.43	1.53	2.79
203729_at	**EMP3**	**Epithelial membrane protein 3**	**2.11**	**2.94**	**1.61**
208997_s_at	UCP2	Uncoupling protein 2	2.09	1.79	2.48
207740_s_at	NUP62	Nucleoporin 62 kDa	2.39	0.93	2.82
222650_s_at	SLC2A4	SLC2A4 regulator	1.14	2.38	2.44
207540_s_at	**SYK**	**Spleen tyrosine kinase**	**0.33**	**3.76**	**0.78**
207714_s_at	**SERPINH1**	**Heat shock protein 47**	**2.85**	**1.58**	**1.59**
221666_s_at	**TMS1**	**Target of methylation-induced silencing 1**	**1.27**	**1.41**	**20.08**
201781_s_at	**AIP**	**Aryl hydrocarbon receptor-interacting protein**	**1.01**	**0.86**	**2.29**

AP-4 (*TFAP4*), Zinc finger protein 36, C3H type-like 2 (*ZFP36L2*), *ARGHDIA*, suppressor of lin-12-like (*SEL1*) and latent-transforming growth factor β-binding protein 3 (*LTBP3*)) by RT-PCR analysis before and after 5-aza-dC treatment in each of the three neuroblastoma cell lines. In each case, expression was consistent with the results of microarray analysis (data not shown).

5′ CpG methylation status for candidate epigenetically inactivated genes

After applying the proposed selection criteria, 21 candidate 'novel epigenetically silenced TSGs' were selected according to the strict criteria (upregulated in two or more cell lines) and a further seven genes previously reported to be methylated in other tumour types and upregulated in at least one cell line were selected for further analysis.

The methylation status of the 5′ promoter region CpG island was determined by direct sequencing of bisulphite-converted DNA. Out of the 21 candidate novel phaeochromocytoma TSGs, 19 (excepting *KRT19* and *CST6*) were completely unmethylated in the three neuroblastoma cell lines and in adult normal adrenal DNA. Sequencing of the *Keratin 19* (*KRT19*), 5′ CpG island revealed heavy methylation in all three cell lines (22/29, 25/29 and 29/29 CpG dinucleotides examined were methylated in SK-N-F1, SK-N-BE and SK-N-DZ respectively). However, the normal adrenal DNA also showed a similar pattern of CpG methylation (24/29 CpG dinucleotides methylated). Direct bisulphite sequencing of *CST6* (Cystatin M) 5′ promoter region revealed methylated CpGs in all three neuroblastoma cell lines (data not shown). The CpG island methylation was also detected in four additional cell lines examined. To confirm the results of the direct sequencing, cell line and normal adrenal DNA was subcloned and 10 clones for each cell line were sequenced. Consistent with the results of direct sequencing, CpG methylation was detected in cell lines

but not in normal human adrenal DNA. However, there was no correlation between the extent *CST6* CpG island methylation and upregulation of *CST6* expression after 5-aza-dC treatment. We then proceeded to analyse *CST6* CpG island methylation in 20 primary tumours (10 neuroblastomas and 10 phaeochromocytomas). None of the tumours showed significant promoter methylation.

We then analysed the seven genes (*OPCML*, Junction plakoglobin (*JUP*), *HOXA9*, *EMP3*, *SYK*, *HSP47* and *TMS1*) that a) had previously been shown to have be implicated in human tumourigenesis and b) demonstrated a >2-fold increase in expression after demethylation in at least one of three neuroblastoma cell lines. The promoter methylation status of *OPCML*, *JUP*, *HOXA9*, *EMP3*, *SYK*, *HSP47* and *TMS1* was analysed by previously published MSP primers in cell lines and in up to 33 phaeochromocytomas and 19 neuroblastomas (see Fig. 1 and Table 3). Three genes (*HSP47*, *OPCML* and *HOXA9*) demonstrated frequent (>10%) CpG island methylation in phaeochromocytoma tumours, but methylation of *EMP3*, although very frequent in neuroblastoma, was rare in phaeochromocytoma. Two CpG islands were examined for *HSP47* methylation, one in the 5′ flanking region of *HSP47* that encompasses a promoter and the second region around its transcriptional start site (Yang *et al*. 2007). In agreement with this previous study, similar patterns of methylation were seen in the two regions,

although the promoter region showed a higher methylation frequency (90 and 52% in neuroblastoma and phaeochromocytoma tumours respectively; see Table 2). Methylation of *EMP3* was relatively specific for neuroblastoma tumours (68 vs 6%, $P=0.000006$), whereas the frequency of *HOXA9* promoter methylation was similar in the two tumour types. *TMS1* and *CTNNG* displayed lower frequencies (<10%) of CpG island promoter methylation in phaeochromocytoma tumours and no promoter region methylation was detected in cell lines or primary tumours at *SYK* (Table 3).

No association was detected between methylation at *OPCML*, *HOXA9* or *HSP47* in individual tumours. There was also no association between methylation at *OPCML or HSP47* and methylation as *CASP 8* or *HIC1* (Margetts *et al*. 2005). To analyse whether the different subtypes of neural crest tumours showed different levels of methylation, we combined data from the current study with additional data reported previously for *FLIP*, *TSP1*, *DcR1*, *DcR2*, *DR4*, *DR5*, *CASP8* and *HIC1* (Margetts *et al*. 2005) and estimated the mean percentage of loci methylated in each tumour sample. There were no significant differences in the mean percentage of loci methylated among neuroblastoma (mean 34.3%), VHL phaeochromocytoma (mean 26.8%) and sporadic phaeochromocytoma (mean 27.4%). We did not find any evidence that a particular gene was differentially methylated in VHL and sporadic phaeochromocytomas. To determine whether there was evidence for a subset of tumours with a CpG island methylator phenotype (CIMP), the distribution of number of methylated loci per tumour was compared with that expected from a Poisson distribution. However, there were no significant differences between the observed and expected distributions ($P=0.3751$).

Figure 1 Methylation-specific PCR (MSP) of *OPCML*, *CTNNG*, *HOXA9*, *EMP3*, *SYK*, *HSP47(a)*, *HSP47(b)* and *TMS1* in neuroblastoma and phaeochromocytoma tumours. Bisulphite-modified DNA was amplified with primers specific for unmethylated (U) and methylated (M) DNA. Sample number above lane. NA, normal adrenal DNA. Sizes of the PCR products are indicated by arrows, U for unmethylated and M for methylated respectively. Positive control is *Sss1*-treated DNA.

Discussion

Phaeochromocytoma and neuroblastoma are both derived from the neural crest and there are overlaps between the regions of allele loss and patterns of TSG methylation observed in the two tumour types. In the absence of a human phaeochromocytoma cell line, we analysed gene expression changes following treatment with a demethylating agent in three neuroblastoma cell lines. Such functional epigenomic screens have proven to be a successful strategy to identify epigenetically inactivated TSGs in a number of different tumour types including oesophageal, pancreatic and prostate (Yamashita *et al*. 2002, Sato *et al*. 2003, Lodygin *et al*. 2005).

Table 2 Summary of gene-specific methylation data in VHL-associated and sporadic phaeochromocytomas and neuroblastoma tumours

	Phaeochromocytoma (% methylated)			Neuroblastoma tumours (% methylated)
	All	VHL	Sporadic	
OPCML	12 (4/33)	10.5 (2/19)	14.3 (2/14)	10.5 (2/19)
CTNNG	3 (1/33)	0 (0/19)	7.1 (1/14)	10.5 (2/19)
HOXA9	17.2 (5/29)	25 (4/16)	7.7 (1/13)	22.2 (4/18)
EMP3	6.1 (2/33)	5.3 (1/19)	7.1 (1/14)	68.4 (13/19)
SYK	0 (0/29)	0 (0/16)	0 (0/13)	0 (0/18)
HSP47 (A)	36.4 (12/33)	36.8 (7/19)	35.7 (5/14)	68.4 (13/19)
HSP47 (B)	51.5 (17/33)	52.6 (10/19)	50 (7/14)	89.5 (17/19)
TMS1	6.9 (2/29)	0 (0/16)	15.4 (2/13)	16.7 (3/18)

Data are percentages and numbers of tumour samples analysed. Methylation of *EMP3* and *HSP47 (A)* and *HSP47 (B)* was significantly more frequent in neuroblastomas than phaeochromocytomas ($P = 0.001$, $P = 0.02$ and $P = 0.005$ respectively).

In addition, using this strategy in RCC cell lines, we identified *HAI-2/SPINT2* as a novel epigenetically inactivated RCC TSG (Morris *et al.* 2005).

Although our selection criteria for investigating 'candidate epigenetically silenced TSGs' would be expected to result in some false negative prediction, the major problem we encountered was the absence of promoter region methylation in many of the genes we analysed. In addition, two genes that did show evidence of promoter methylation in neuroblastoma cell lines did not prove to be methylated in primary tumours. Whilst these results were disappointing, they are not unique to neuroblastoma cell lines. Thus, in a similar study of four RCC cell lines, we identified *SPINT2* as a novel epigenetically inactivated renal TSG. However, analysis of a further 60 genes that were differentially expressed after demethylation revealed that only six (four of which had been reported previously) demonstrated primary tumour-specific promoter methylation (Morris *et al.* 2008). Thus, this approach has a relatively low specificity for identifying methylated TSGs. Analysis of a larger number of genes might have led to the identification of novel TSGs, but reducing the stringency of the selection criteria would also probably further lower specificity. A more effective plan of investigation might be to combine 'functional epigenomics' experiments with strategies to directly identify methylated DNA (e.g. MeDIP), as this should help exclude genes whose expression is upregulated after demethylation but do not demonstrate promoter methylation (Wilson *et al.* 2006). Nevertheless, it is possible that candidate TSGs that are unmethylated but upregulated by demethylation may prove to be downstream of epigenetically inactivated TSGs. Thus, we used the Oncomine (http://www.oncomine.org/) data analysis tool to interrogate gene expression data reported by Asgharzadeh *et al.* (2006) for 19 genes that were upregulated by demethylation but did not show detectable CpG methylation (*SNN*, *RASD1*, *ARPC4*, *PTK2B*, *TFAP4*, *ZFP36L2*, *ARHGDIA*, suppressor of lin-12-like (*SEL1L*), *LTBP3*, smoothelin (*SMTH*), ST3 β-galactoside α-2,3-sialyltransferase 2 (*ST3GAL2*), v-rel reticuloendotheliosis viral oncogene homologue A (*RELA*), tripartite motif-containing 3 (*TRIM3*), chromodomain helicase DNA-binding protein 5 (*CHD5*), *PRKA2R*, *AK1*, *UCP2*, *NUP62*, *SLC2A4*). Out of 19 genes, 4 (adenylate kinase 1; *AK1*, *TFAP4*, *SNN* and *SEL1L*) were differentially expressed levels between relapsing and non-relapsing tumours. In addition, *CHD5* has been identified as a candidate 1p36 neuroblastoma TSG (Bagchi *et al.* 2007).

There was a higher frequency of promoter methylation in primary tumours among the seven genes that had been previously implicated in human tumourigenesis and were selected according to the less strict criteria (upregulated in one or more cell lines). Three of these genes, *HSP47*, *HOXA9* and *OPCML* were methylated in >10% of phaeochromocytomas.

Both heat-shock protein 47 (*HSP47*) and *OPCML* map to 11q (11q13.5 and 11q25 respectively) and chromosome 11q allele loss is frequent in both tumour types (Yokogoshi *et al.* 1990, Sun *et al.* 2006, George *et al.* 2007). *HSP47* encodes a collagen-specific molecular chaperone and is essential for the production and maturation of collagens I and IV (Sauk *et al.* 2005). Type I collagen negatively regulates cell proliferation and is deficient in aggressive neuroblastoma tumours (Yang *et al.* 2004). Previously, expression of *HSP47* was reported to be upregulated after treatment with 5-aza-dC in the neuroblastoma cell line NB2-W-S and promoter region methylation was detected in

Table 3 Individual tumour–methylation patterns

Tumour type and number	Gene								MI
	OPCML	CTNNG	HOX9A	EMP3	SYK	HSP47(A)	HSP47 (B)	TMS1	
PV 03	U	U	U	U	U	U	M	U	0.125
PV 04	U	U	U	U	U	M	M	U	0.25
PV 05	U	U	M	U	U	M	M	U	0.375
PV 06	U	U	U	U	U	U	U	U	0
PV 07	U	U	U	U	U	U	M	U	0.125
PV 08	U	U	U	U	U	U	U	U	0
PV 09	U	U	U	U	U	M	M	U	0.25
PV 10	U	U	U	U	U	M	M	U	0.25
PV 11	U	U	ND	U	ND	U	U	ND	0
PV 12	U	U	ND	U	ND	M	M	ND	0.25
PV 13	U	U	M	U	U	U	U	U	0.125
PV 15	U	U	U	U	U	U	U	U	0
PV 16	U	U	U	U	U	M	M	U	0.25
PV 17	U	U	M	U	U	U	U	U	0.125
PV 18	U	U	U	U	U	U	U	U	0
PV 19	M	U	U	U	U	U	U	U	0.125
PV 21	U	U	M	U	U	U	U	U	0.125
PV 22	M	U	ND	U	ND	U	M	ND	0.25
PV 23	U	U	U	M	U	M	M	U	0.375
PS 03	U	M	M	M	U	M	M	M	0.75
PS 05	M	U	U	U	U	U	U	U	0.125
PS 06	M	U	U	U	U	M	M	U	0.375
PS 07	U	U	U	U	U	U	U	M	0.125
PS 08	U	U	U	U	U	U	U	U	0
PS 09	U	U	U	U	U	U	M	U	0.125
PS 11	U	U	ND	U	ND	U	U	ND	0
PS 12	U	U	U	U	U	M	M	U	0.25
PS 13	U	U	U	U	U	U	U	U	0
PS 14	U	U	U	U	U	U	U	U	0
PS 15	U	U	U	U	U	M	M	U	0.25
PS 16	U	U	U	U	U	M	M	U	0.25
PS 17	U	U	U	U	U	U	M	U	0.125
PS 18	U	U	U	U	U	U	U	U	0
NB 01	U	U	M	M		M	M	ND	0.67
NB 03	U	M	U	U	U	U	M	U	0.25
NB 04	U	U	U	U	U	U	M	U	0.125
NB 05	U	U	U	U	U	U	M	U	0.125
NB 06	U	U	M	M	U	M	M	U	0.5
NB 07	U	U	U	M	U	M	M	M	0.375
NB 08	U	U	U	U	U	U	U	U	0
NB 09	U	U	U	M	U	M	M	U	0.375
NB 10	U	U	U	M	U	M	M	U	0.375
NB 11	M	U	U	M	U	M	M	U	0.5
NB 12	U	M	U	U	U	U	M	U	0.25
NB 13	U	U	U	M	U	M	M	U	0.375
NB 14	U	U	U	M	U	M	M	U	0.375
NB 15	U	U	U	M	U	M	M	U	0.375
NB 16	U	U	U	M	U	M	M	U	0.375
NB 17	U	U	M	M	U	M	M	M	0.625
NB 18	U	U	M	M	U	M	M	M	0.625
NB 19	U	U	U	U	U	U	U	U	0
NB 20	M	U	ND	M	ND	M	M	ND	0.8

NB, neuroblastoma; PS, sporadic phaeochromocytoma; PV, VHL, associated phaeochromocytoma; U, unmethylated; M, methylated; ND, not determined (failed); MI, methylation index (number of methylated genes/number genes analysed).

neuroblastoma cell lines and 4/7 primary neuroblastoma tumours analysed (Yang *et al.* 2004). In addition, expression of *HSP47* correlated with that of collagen I and IV. We have confirmed and extended the analysis of *HSP47* promoter methylation in neuroblastoma and demonstrated that the *HSP47* promoter is methylated in ∼50% of phaeochromocytomas.

HOXA9 promoter region methylation was detected in about one-fifth of neuroblastoma and phaeochromocytomas. Several *Hox* genes have been reported to be methylated in human cancers, e.g. *HOXB13* and *HOXA5*, in renal and breast cancer respectively (Okuda *et al.* 2006, Piotrowski *et al.* 2006). *HOXA9* promoter methylation was identified previously in lung cancer (Rauch *et al.* 2007) and in neuroblastoma (Alaminos *et al.* 2004), but not in phaeochromocytoma. Previously, we found significant differences in the frequency of *HIC1* and *CASP8* methylation between VHL-associated and sporadic phaeochromocytomas, but in this study we did not identify any further differentially methylated genes.

OPCML (OPCML/cell adhesion molecule-like) encodes a member of the IgLON subfamily in the immunoglobulin protein superfamily and was identified as a candidate TSG following the identification of extensive promoter methylation in ovarian cancer (Sellar *et al.* 2003). Ectopic expression of the OPCML gene product suppressed cell growth *in vitro* and tumour growth *in vivo* in nude mice models. Promoter methylation associated silencing of *OPCML* has also been demonstrated in hepatocellular carcinomas (Liu *et al.* 2006) and gliomas and other brain tumours (including medulloblastomas) (Reed *et al.* 2007). To our knowledge, *OPCML* promoter methylation has not previously been reported in neuroblastoma or phaeochromocytoma.

EMP3 is a peripheral myelin protein involved in cell proliferation and cell–cell interactions. Previously *EMP3* expression was reported to be downregulated in glioma and neuroblastoma tumours, and *EMP3* promoter methylation was detected in 24% of neuroblastomas (Alaminos *et al.* 2005). However, although we confirmed frequent *EMP3* promoter methylation in neuroblastoma, *EMP3* methylation did not appear to make a significant contribution to phaeochromocytoma tumourigenesis.

Infrequent, or absent, promoter methylation was found for *CTNNG* (γ-catenin, also known as *JUP*) and *SYK* in phaeochromocytoma. Although Amitay *et al.* (2001) reported reduced expression of *CTNNG* in 9/20 neuroblastoma tumours, we observed promoter methylation in only 11% of neuroblastoma tumours and did not detect promoter methylation in the cell lines that demonstrated

an increase expression of *CTNNG* after 5-aza-dC treatment. This suggests that promoter methylation of *CTNNG* does not play a large role in its regulation of expression in neural crest tumours. *SYK* (spleen tyrosine kinase) and *TMS1* (also known as *ASC*, apoptosis speck-like protein containing CARD) have been reported to be methylated in 60 and 80% of neuroblastoma cell lines respectively (Alaminos *et al.* 2004), our results suggest that methylation is less frequent in neuroblastoma primary tumours and is infrequent (<10%) in phaeochromocytoma. The CIMP methylator phenotype is well described in a subset of colorectal cancers (Weisenberger *et al.* 2006). However, we did not find evidence of a CIMP subgroup in neural crest tumours and mean methylation index did not differ between neuroblastomas, VHL and sporadic phaeochromoctromas. Our findings demonstrate that many genes that are methylated in neuroblastoma are also methylated in phaeochromocytoma. Identification of methylated genes in neural crest tumours will provide potential biomarkers and can provide insights into the molecular pathogenesis of tumour development in these disorders.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

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