Acquired hypermethylation of the P16INK4A promoter in abdominal paraganglioma: relation to adverse tumor phenotype and predisposing mutation

Nimrod B Kiss[1,2], Andreas Muth[3], Adam Andreasson[1,2], C Christofer Juhlin[2,4], Janos Geli[1], Martin Bäckdahl[1], Anders Höög[4], Bo Wängberg[3], Ola Nilsson[5], Håkan Ahlman[3] and Catharina Larsson[1,2]

[1]Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital, CCK R8:04, SE-171 76 Stockholm, Sweden
[2]Cancer Centre Karolinska, Karolinska University Hospital, Stockholm, Sweden
[3]Department of Surgery, Sahlgrenska University Hospital, Göteborg, Sweden
[4]Department of Oncology–Pathology, Karolinska Institutet, Stockholm, Sweden
[5]Department of Pathology, Sahlgrenska University Hospital, Göteborg, Sweden

Correspondence
should be addressed to
N B Kiss at Department of
Molecular Medicine and
Surgery, Karolinska Institutet,
Karolinska University Hospital
Email
nimrod.kiss@ki.se

Abstract

Recurrent alterations in promoter methylation of tumor suppressor genes (TSGs) and *LINE1* (*L1RE1*) repeat elements were previously reported in pheochromocytoma and abdominal paraganglioma. This study was undertaken to explore CpG methylation abnormalities in an extended tumor panel and assess possible relationships between metastatic disease and mutation status. CpG methylation was quantified by bisulfite pyrosequencing for selected TSG promoters and *LINE1* repeats. Methylation indices above normal reference were observed for *DCR2* (*TNFRSF10D*), *CDH1*, *P16* (*CDKN2A*), *RARB*, and *RASSF1A*. *Z*-scores for overall TSG, and individual TSG methylation levels, but not *LINE1*, were significantly correlated with metastatic disease, paraganglioma, disease predisposition, or outcome. Most strikingly, *P16* hypermethylation was strongly associated with *SDHB* mutation as opposed to *RET*/MEN2, *VHL*/VHL, or NF1-related disease. Parallel analyses of constitutional, tumor, and metastasis DNA implicate an order of events where constitutional *SDHB* mutations are followed by TSG hypermethylation and 1p loss in primary tumors, later transferred to metastatic tissue. In the combined material, *P16* hypermethylation was prevalent in *SDHB*-mutated samples and was associated with short disease-related survival. The findings verify the previously reported importance of *P16* and other TSG hypermethylation in an independent tumor series. Furthermore, a constitutional *SDHB* mutation is proposed to predispose for an epigenetic tumor phenotype occurring before the emanation of clinically recognized malignancy.

Key Words
- Molecular genetics
- Gene regulation
- Metastasis
- Adrenal medulla
- Endocrine therapy

Endocrine-Related Cancer
(2013) **20**, 65–78

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Introduction

Pheochromocytomas are catecholamine-secreting tumors of the chromaffin cells of the adrenal medulla. Extra-adrenal abdominal paragangliomas (here referred to as abdominal paraganglioma or paraganglioma) are related to neuroendocrine tumors in chromaffin cells distributed along the sympathetic ganglia throughout the abdomen. There is an increasing appreciation for the hereditary background of these tumors; indeed, known predisposing gene variations are present in more than 25% of pheochromocytoma and paraganglioma patients (Neumann *et al*. 2002). Four syndromes are closely associated with pheochromocytomas and paragangliomas: MEN2, VHL, neurofibromatosis type 1, and familial paraganglioma syndrome types 1, 3, and 4, which are caused by constitutional mutations in the *RET*, *VHL*, *NF1*, and *SDHD*, *SDHC*, and *SDHB* genes respectively (Elder *et al*. 2005). Recently, constitutional mutations of *SDHAF2* (*SDH5*) and *SDHA* were also identified in paraganglioma and of *TMEM127* and *MAX* in pheochromocytoma (Hao *et al*. 2009, Burnichon *et al*. 2010, Qin *et al*. 2010, Yao *et al*. 2010, Comino-Méndez *et al*. 2011).

Others and we have previously shown that promoter hypermethylation in tumor suppressor genes (TSGs) is a prominent feature of pheochromocytoma and paraganglioma (Cascon *et al*. 2004, Margetts *et al*. 2005, Geli *et al*. 2007, Geli *et al*. 2008, Kiss *et al*. 2008, Muscarella *et al*. 2008, Sandgren *et al*. 2010*a*). P16 (CDKN2A) and its novel transcript variant P16G are important tumor suppressors that act through the retinoblastoma pathway to regulate the cell cycle (Lin *et al*. 2007). Knocking out the mouse equivalent of the INK4A locus, Ink4a/Arf, causes a substantial increase in the severity of the disease phenotype in pheochromocytoma-prone mice (You *et al*. 2002). While this region is rarely lost in human pheochromocytomas and paragangliomas, we found that hypermethylation of the residing *P16* gene is strongly associated with malignancy – 4/5 cases with *P16* hypermethylation were classified as malignant, while only 1/44 tumors without evidence of malignancy harbored *P16* hypermethylation (Kiss *et al*. 2008). We further found concerted hypermethylation in the promoters of the *CDH1*, *DCR2* (*TNFRSF10D*), *p16^{INK4A}* (*CDKN2A*), *RASSF1A*, and *RARB* genes in the same panel of 55 pheochromocytomas and paragangliomas (Geli *et al*. 2008). Coordinated hypermethylation of multiple TSGs was observed in agreement with a CpG island methylator phenotype (CIMP) originally described for colon cancer (Toyota *et al*. 1999). Interestingly, CIMP was associated with malignancy (4/5 cases with CIMP had developed metastases) and with *SDHB* mutation (*SDHB* mutation was detected in 4/5 cases with CIMP, Geli *et al*. (2008) and Kiss *et al*. (2008)).

In this study, we aimed to evaluate the possibility that predisposing mutation and TSG hypermethylation are associated events and to substantiate the evidence that TSG hypermethylation is indicative of malignant behavior. We have therefore characterized TSG promoter hypermethylation in relation to global CpG methylation, analogized by assessing methylation in the *LINE1* (*L1RE1*) retrotransposon element, and in relation to disease predisposition in an extended tumor panel of pheochromocytomas and paragangliomas. Notably and as our previous findings indicated a presence of CIMP in strong association with malignant paragangliomas with *SDHB* mutation (Geli *et al*. 2008, Kiss *et al*. 2008), the present panel included additional cases with known mutation in predisposing genes and/or associated syndromes, paragangliomas, and metastatic disease. Furthermore, comparisons were made with non-tumorous material with regard to genetic and epigenetic events in a subset of cases. Our aims were to i) verify the occurrence of TSG hypermethylation in an independent tumor series; ii) assess the temporal relation of methylation to tumor development; iii) determine the chronological relation to genetic alterations; iv) evaluate the relation to mutations in predisposing genes; and v) assess associations to metastatic disease.

Materials and methods

Patients and clinical samples

All samples were collected with informed consent and ethical approval from patients surgically treated for pheochromocytoma or abdominal paraganglioma at Sahlgrenska University Hospital, Göteborg or Karolinska University Hospital, Stockholm, Sweden (Table 1). Fresh frozen tumor samples and matching non-tumor tissues were obtained from the respective endocrine Biobanks and matching leukocytes from peripheral venous samples. In this study, tumors were classified according to the World Health Organization criteria (DeLellis 2004, Tischler 2008) whereby only cases with metastases were regarded as malignant.

Tumor series A (Tables 1 and 2) constituted of 38 primary tumors and two metastases (Khorram-Manesh *et al*. 2005, Wängberg *et al*. 2006, Muth *et al*. 2012) was analyzed for promoter methylation and *SDHB/D* mutation

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

Table 1 Clinical and genetic details for pheochromocytoma/paraganglioma cases.

Parameter	This study		
Phenotype	**Series A**	**Series B[a]**	**Series A+B**
No of cases			
Patients	39	54	93
Tumors	40	56	96
Samples studied			
Blood/normal tissues	–	5	
Primary tumors	38	54	92
Metastases	2	2	4
Gender			
Female	20	31	51
Male	18	23	41
Age at diagnosis			
Range (years)	17–81	13–77	13–81
Syndrome/mutation			
SDHB	6	4	10
MEN2/*RET*	9	3	12
NF1	5	2	7
VHL/*VHL*	2	1	3
Diagnosis			
Pheochromocytoma	27	43	70
Paraganglioma	12	11	23
Metastasized	7	7	14
Without metastasis	32	47	79
Bilateral/multiple	7	6	13
Survival			
DOD	4	2	6
DOC	7	6	13
A	27	45	72

DOD, dead of disease; DOC, dead of other cause; A, alive, no reported disease.
[a]Data for tumors from Geli *et al*. (2008) and Kiss *et al*. (2008).

in this study (Table 2). For Series B (Table 1), clinical/genetic details and methylation status for tumor samples have been previously published (Edström Elder *et al*. 2003, Geli *et al*. 2008, Kiss *et al*. 2008, Sandgren *et al*. 2010*b*). In this study, mutation screenings and methylation quantifications in Series B were carried out in matching normal samples from a subset of cases (Supplementary Table S1, see section on supplementary data given at the end of this article). Histopathologically evaluated normal adrenal medullary DNA samples purchased from Clinomics (Watervliet, NY, USA) were used as non-tumor references in methylation analyses.

DNA extractions

Genomic DNA was extracted from tissue and blood samples applying ChargeSwitch gDNA Mini Tissue Kit (Invitrogen/Life Technologies Corporation). A few of the non-tumor reference cases were extracted using a standard method with proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation. DNA quality and concentrations were assessed using a NanoDrop Spectrophotometer (ND-1000).

Sequencing of the *SDHB* and *SDHD* genes

The coding exons and exon–intron junctions of the *SDHB* and *SDHD* genes were sequenced in tumor and normal DNA samples. *SDHB* sequences were amplified by PCR as eight different fragments and *SDHD* in four different reactions under previously described experimental conditions (Baysal *et al*. 2000, Benn *et al*. 2003, Castellano *et al*. 2006). Primer sequences and amplification details are given in Supplementary Table S2, see section on supplementary data given at the end of this article. PCR amplicons were purified by Exozap-IT (USB, GE Healthcare, Pittsburgh, PA, USA) and sequenced at the KIGene facility at KI, Stockholm, Sweden, using the ABI 3730 DNA Analyzer system (Applied Biosystems). Sequences were subsequently analyzed with the CodonCode and SeqScape Software (Applied Biosystems). Detected sequence alterations were verified and re-sequenced by forward and reverse sequencing of the involved exons.

SDHB was sequenced in 22 tumors from Series A (AG-1, -2, -6, -9, -10, -13, -14, -16, -18a, -18b, and -20; AS-21, -23, -28, -32, -33, -34, -35, -37, -38, -39, and -40). These included all paragangliomas and all pheochromocytomas previously described as malignant from Series A. Further, *SDHB* was sequenced in eight tumors from Series B (BS-7, -26, -30, -33, -36, -41, -44, and -45), including paragangliomas and malignant pheochromocytomas. In addition, non-tumorous DNA was sequenced in five cases in Series B with previously reported *SDHB* tumor mutations (Kiss *et al*. 2008) to assess whether mutations were constitutional or not. *SDHD* was sequenced in ten tumors (AS-23, -28, -32, -33, -34, -35, -37, -38, -39, and -40) including samples previously described as malignant in Series A and one tumor in Series B (BS-2) that featured CIMP but where no *SDHB* mutation could be detected (Geli *et al*. 2008, Kiss *et al*. 2008).

Mutation status of predisposing genes

Cases with a hereditary form of the disease were identified by mutation screening of constitutional DNA or in some cases based on the clinical presentation as reported in Edström Elder *et al*. (2003) and Muth *et al*. (2012). In addition, cases with *SDHB* mutations were identified by sequencing of tumor DNA and by verification of

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

Table 2 Results from methylation quantifications of promoter regions in tumor Series A.

						DCR2 (% meth)		*CDH1* (%)		*NORE1A* (%)		*P16* (%)		*RARB* (%)		*RASSF1A* (%)		*LINE-1* (%)	
Case no.	**Tumor type**	**Metastatic disease**	**Mutation/ syndrome**	**CIMP type**	***Z*-score[a]**	**MetI**	**Range**	**MetI**	**Range**	**MetI**	**Range**	**MetI**	**Range**	**MetI**	**Range**	**MetI**	**Range**	**MetI**	**Range**
AG-1	Pheo	No	MEN2	–	−0.2	4	1–8	2	1–4	2	1–6	1	1–2	3	2–4	17	6–**36**	46	37–54
AG-2	Paraga	Met	*SDHB*	–	0.5	15	11–26	**11**	4–**18**	3	1–7	**10**	8–**13**	4	3–7	7	7–8	75	74–77
AG-3	Pheo	No	*RET*	–	−0.4	3	1–7	3	1–**15**	2	1–6	1	1–1	3	2–4	2	1–3	68	65–73
AG-4	Pheo	No	NF1	–	0.1	21	6–**33**	4	1–6	2	2–5	2	0–9	3	0–5	10	7–15	75	71–77
AG-5	Pheo	No	NF1	–	−0.4	4	2–9	3	1–6	2	1–6	1	1–2	–	–	3	2–3	71	66–75
AG-6	Paraga	No	*SDHB*	–	0.2	12	6–20	8	2–**20**	3	1–6	9	3–**17**	2	1–3	11	5–20	77	75–78
AG-7	Pheo	No	NF1	–	−0.2	13	4–22	4	1–7	2	1–5	1	1–2	2	1–3	7	3–11	74	70–76
AG-8	Pheo	No	*RET*	–	−0.5	2	1–4	3	1–5	2	1–5	1	1–2	2	1–3	2	1–3	76	71–81
AG-9	Paraga	Met	*SDHB*	CIMP	2.8	**49**	22–**64**	4	1–7	6	3–**11**	**50**	**40–57**	**21**	4–**37**	**42**	**39–47**	68	64–74
AG-10	Pheo	No	–	–	0.1	6	1–15	4	1–**20**	2	1–5	1	0–2	2	0–3	**35**	30–**42**	72	70–76
AG-11	Pheo	No	*VHL*	–	−0.3	4	1–5	4	1–7	2	1–4	1	1–2	3	2–3	2	1–3	80	76–83
AG-12	Pheo	No	*VHL*	–	−0.3	6	1–12	5	1–10	2	1–6	1	1–1	2	1–3	1	1–1	77	75–78
AG-13	Paraga	No	–	–	0.5	25	6–**43**	5	2–8	2	1–5	**29**	**15–49**	2	0–4	12	8–16	78	75–81
AG-14	Paraga	Met	*SDHB*	CIMP	2.8	**50**	**34–58**	**24**	5–**53**	8	3–**27**	**40**	**32–47**	2	2–4	28	25–**33**	79	78–79
AG-15	Pheo	No	NF1	–	−0.3	3	1–5	4	2–5	2	1–4	1	1–2	–	–	13	4–**33**	73	68–76
AG-16	Paraga	No	–	–	−0.4	3	1–4	3	2–5	2	1–6	1	1–1	–	–	8	4–15	76	72–78
AG-17	Pheo	No	*RET*	–	0.0	7	2–13	5	2–8	4	1–**11**	1	1–2	4	3–5	3	2–4	77	75–79
AG-18a	Paraga	Met	–	–	0.6	13	7–18	5	2–8	2	1–6	**21**	**18–25**	–	–	30	27–**33**	72	70–73
AG-18b	Paraga		–	–	–	16	3–**34**	4	2–8	4	2–**11**	1	1–2	3	2–6	3	2–4	76	73–79
AG-19	Pheo	No	*RET*	–	−0.3	5	2–11	2	1–5	3	1–**11**	4	1–**14**	2	0–3	2	1–3	70	66–74
AG-20	Paraga	No	*SDHB*	CIMP	1.3	**45**	17–**69**	4	1–6	2	1–7	**40**	14–59	3	2–5	**32**	15–**51**	64	61–70
AS-21	Paraga	Met	*SDHB*	–	0.6	27	6–**48**	6	2–8	2	1–6	8	4–**12**	8	2–**25**	17	8–28	76	70–74
AS-22	Pheo	No	*RET*	–	−0.4	4	0–11	3	0–7	2	0–8	2	0–6	0	0–0	9	1–30	62	57–66
AS-23	Pheo	No	–	–	−0.3	2	0–14	8	0–**29**	2	0–6	0	0–1	1	0–4	9	0–21	64	57–70
AS-24	Pheo	No	NF1	–	−0.6	5	2–11	5	3–8	0	0–4	2	0–4	1	0–4	2	0–4	61	58–63
AS-25	Pheo	No	MEN2	–	−0.7	2	0–6	1	0–5	1	0–3	0	0–1	0	0–3	8	6–11	51	46–57
AS-26	Pheo	No	*RET*	–	−0.4	11	4–17	2	0–7	1	0–4	2	1–3	2	0–5	7	1–20	59	57–61
AS-27	Pheo	No	*RET*	–	−0.5	1	0–3	6	0–**45**	1	0–6	0	0–1	0	0–4	0	0–1	63	58–68
AS-28	Pheo	No	–	–	−0.3	5	2–11	6	3–9	1	0–4	2	0–4	3	0–9	2	0–4	63	59–65
AS-29	Pheo	No	–	–	−0.3	10	0–**47**	2	0–4	2	0–5	2	1–4	2	0–8	11	4–**34**	59	53–63
AS-30	Pheo	No	–	–	−0.5	1	0–4	–	–	1	0–5	0	0–1	0	0–4	12	4–24	61	58–65
AS-31	Pheo	No	–	–	0.2	16	3–**58**	6	0–**23**	1	0–5	1	0–1	0	0–4	**38**	22–**50**	56	53–58
AS-32	Pheo	No	–	–	−0.2	2	0–5	**14**	4–**34**	1	0–4	1	0–3	0	0–0	3	3–5	63	58–67
AS-33	Pheo	No	–	–	−0.5	11	0–24	3	0–7	1	0–4	1	0–1	0	0–4	1	1–2	66	64–67
AS-34	Pheo	No	–	–	−0.4	19	5–**35**	3	0–5	1	0–5	1	1–2	1	0–3	8	5–11	64	60–67
AS-35	Pheo	No	–	–	−0.5	1	0–4	4	0–**15**	1	0–5	2	0–8	1	0–6	1	1–1	60	56–63
AS-36	Pheo	No	–	–	−0.3	9	0–**32**	2	0–3	1	0–5	0	0–1	0	0–0	24	4–**39**	54	47–57
AS-37	Paraga	No	–	–	−0.4	2	0–5	3	0–7	2	0–7	1	0–5	1	0–5	12	0–**38**	68	65–74
AS-38	Paraga	Met	–	–	−0.2	3	1–6	–	–	3	0–7	1	0–1	0	0–2	14	6–17	53	49–55
AS-39	Paraga	No	–	–	−0.3	3	0–5	7	3–10	1	0–4	2	0–3	4	2–6	0	0–1	66	64–67
Reference values Cutoff (%)						>30	>30	>10	>10	>10	>10	>10	>10	>10	>10	>30	>30		

meth, methylation; range, minimum–maximum value detected at an individual. CpG G-18a and G-18b are primary tumor and metastasis from the same patient.

Table 3 Correlations between *Z*-scores and clinical/genetic characteristics.

Promoter	Gender	Age at surgery	Mutation	Tumor type	Metastatic disease	Outcome	CIMP
Series A							
Mean *Z*-score	NS	NS	<0.007	<0.0002	<0.0004	0.01	<0.005
DCR2	NS	NS	<0.01	0.01	<0.02	NS	<0.005
CDH1	NS	NS	NS	NS	<0.04	NS	NS
NORE1A	NS	NS	NS	0.002	<0.006	0.01	0.01
P16	0.04	NS	0.005	0.001	<0.05	NS	<0.005
RARB	NS	NS	0.02	<0.005	NS	NS	<0.05
RASSF1A	NS	NS	0.005	<0.008	0.002	0.009	0.01
LINE1	0.003	<0.05	NS	NS	NS	NS	NS
Series A+B							
Mean *Z*-score	NS	NS	0.0002	<0.00001	0.0006	<0.0009	<0.000001
DCR2	<0.002	NS	0.0003	<0.0002	0.01	<0.02	<0.000001
CDH1	NS	NS	<0.01	0.03	0.04	0.03	NS
NORE1A	NS	NS	NS	0.01	0.005	<0.04	0.02
P16	NS	NS	0.0002	0.00002	0.002	<0.02	<0.000001
RARB	NS	NS	0.003	<0.008	<0.05	NS	<0.0003
RASSF1A	NS	NS	0.0002	0.0004	<0.0004	<0.0008	<0.000001
LINE1	<0.002	NS	NS	NS	NS	NS	NS

NS, not significant.

constitutional mutations in samples from blood or normal tissues. Results from tumor analyses have been published for Series B in Geli *et al*. (2008) and Kiss *et al*. (2008).

Pyrosequencing of promoter regions

Promoter methylation density was quantified in tumor and normal DNA samples for the TSGs *DCR2*, *CDH1*, *NORE1A*, *P16*, *RARB*, and *RASSF1A* and for *LINE1* repeat elements. All assays were performed according to the PyroMark Assay Database, with the exception of *P16* and *LINE1* that were available as analysis kits from Qiagen. Genomic DNA was sodium bisulfite modified with the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA, USA). Target regions were amplified by PCR from 25 to 50 ng DNA using 0.2 mM of the forward and reverse primers detailed in Supplementary Table S2, 0.2 mM dNTPs, 1.6 units of HotStarTaq, 10× PCR buffer (Qiagen), and for *DCR2* 3.0 mM $MgCl_2$ (Qiagen). Subsequent pyrosequencing was carried out using sequencing primers and annealing temperatures listed in Supplementary Table S2 and Biotage/Qiagen PyroMark equipment (Qiagen).

Methylation indices (MetI) were calculated as the mean of all CpGs assayed for a TSG, and in addition, methylation densities at individual CpGs were considered. Cutoffs for hypermethylation were based on previous analyses of normal adrenal medullary samples (Geli *et al*. 2008, Kiss *et al*. 2008, Kiss *et al*. 2012). Hence, cutoffs for hypermethylation were set at >10% for *CDH1*, *NORE1A*, *P16*, and *RARB* and at >30% for *DCR2* and *RASSF1A*, the elevated cutoffs being based on observations of higher levels of intrinsic methylation in normal samples for the latter two.

Statistical analyses

To allow comparison between samples in relation to various parameters, *Z*-scores were calculated for each sample with reference to each TSG, mean of all TSGs, and *LINE1* in the following way: (mean CpG methylation density for each sample – mean methylation density for that promoter in the tumor panel)/S.D. of that methylation density. This negated large differences in mean MetIs between different genes and allowed direct comparison of gene methylation levels, which are expressed as S.D.s from the mean for the particular gene. STATISTICA 10.0 Software (Statsoft, Inc., Tulsa, OK, USA) was used for all statistical calculations (Supplementary Table S3, see section on supplementary data given at the end of this article). *Z*-scores were compared for cases with different clinical and genetic features – summarized in Table 3 – including age, tumor type, predisposing mutation/syndrome, metastasis, outcome, and relation to CIMP. Mann–Whitney *U* test and Kruskal–Wallis one-way ANOVA test were used to compare groups of continuous data; Fisher's exact test was used for comparisons of categorical data, and Spearman's rank order correlation was used to assess correlations between continuous data sets. Survival curves for hypermethylated and unmethylated cases in Series

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

A+B were compared by log-rank test for *P16* and *RASSF1A*, and results were illustrated graphically by Kaplan–Meier plots. *P* values <0.05 were considered as statistically significant. One of the metastases from Series A (AG-18b) was also represented by its primary tumor and was therefore excluded from the statistical calculations.

Results

Promoter hypermethylation in *DCR2*, *CDH1*, *NORE1A*, *P16*, *RARB*, and *RASSF1A*

Thirty-eight primary tumors and two metastases in Series A were assayed for promoter hypermethylation in multiple CpGs of *DCR2*, *CDH1*, *NORE1A*, *P16*, *RARB*, and *RASSF1A* by pyrosequencing. Three primary tumors fulfilled the criteria of CIMP phenotype, i.e. exhibited increased MetI for three or more genes (AG-9, -14, and -20; Table 2). Hypermethylation with MetI above cutoff was observed for all genes except *NORE1A*, and in addition, some tumors exhibited increased methylation at ≥ 1 CpG without raising the MetI above cutoff (Table 2). Increased MetI was identified in three primary tumors for *DCR2*, three tumors for *CDH1*, five tumors for *P16*, one tumor for *RARB*, and five tumors for *RASSF1A*. In addition, hypermethylation at single CpG(s) was observed for *DCR2* (in four additional primary tumors), *CDH1* (seven tumors), *NORE1A* (five tumors), *P16* (four tumors), and *RASSF1A* (six tumors). In the two metastases, hypermethylation was not observed in regard to

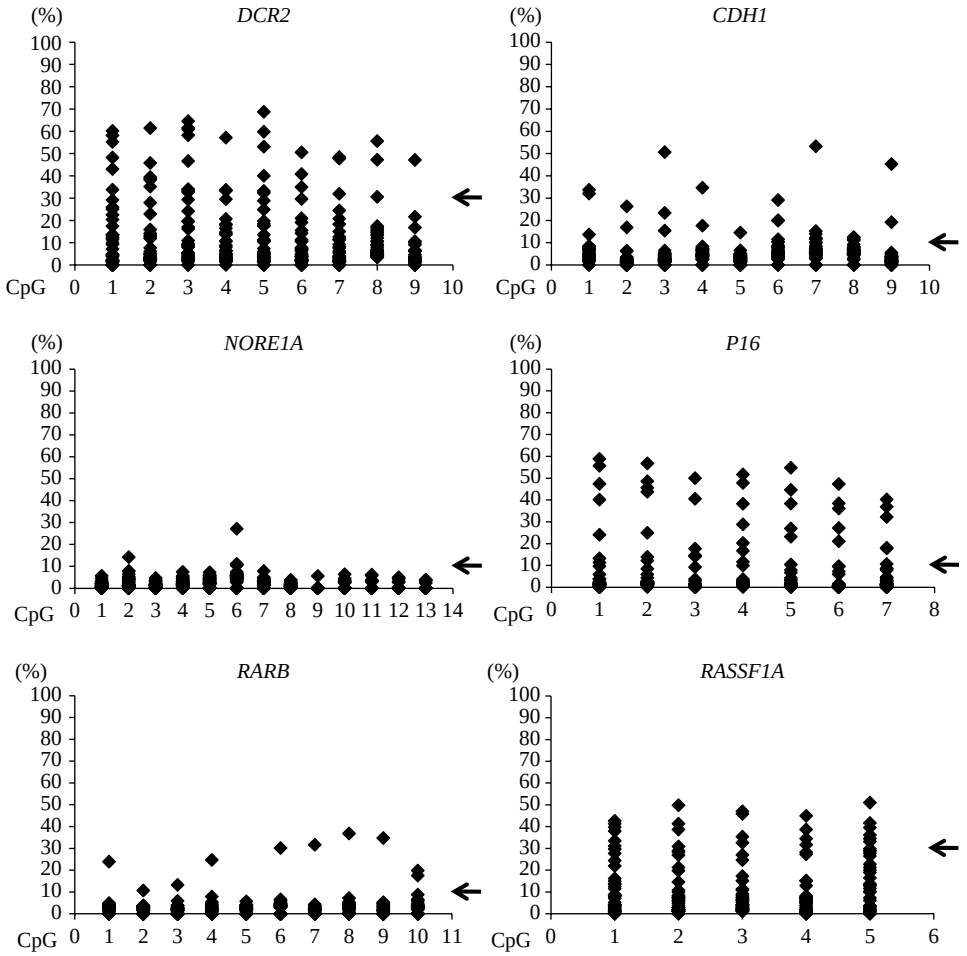

Figure 1

Methylation densities of promoter regions for individual tumor suppressor genes measured by pyrosequencing for pheochromocytomas and paragangliomas in Series A. Scatterplots illustrate methylation levels (%) at individual CpG sites assayed in each tumor sample. Cutoff levels for hypermethylation determined from normal adrenal medulla are indicated by arrows for *DCR2* (>30%), *CDH1* (>10%), *NORE1A* (>10%), *P16* (>10%), *RARB* (>10%), and *RASSF1A* (>30%).

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

MetI, although hypermethylation above cutoff was observed at single CpGs in several of the genes (AG-18b and AS-21; Table 2).

For all promoters, the distribution of methylation densities was relatively even without obvious involvement of single CpGs (Fig. 1). However, the methylation levels varied largely between genes; *DCR2*, *P16*, and *RASSF1A* showed the highest levels, while methylation was less pronounced for *CDH1*, *NORE1A*, and *RARB* (Fig. 1). For *DCR2* and *RASSF1A*, the higher densities also coincided with somewhat higher levels of methylation in normal references, while this was not the case for *P16*.

Association of TSG hypermethylation with clinical and genetic features

Methylation densities in cases with different clinical and genetic features from Series A and B were compared using *Z*-scores calculated for the mean of all TSGs as well as for each individual TSG. Importantly, higher mean *Z*-score was significantly associated with mutation (*SDHB*), tumor type (paraganglioma), metastatic disease, death of disease, and CIMP (Fig. 2; Table 3). High *Z*-scores for individual TSG promoters were also significantly associated with different clinical and genetic features including *SDHB* mutation (all save *NORE1A*), paraganglioma tumor type (all), metastasis (all), and death of disease (all except *RARB*, with *RASSF1A* being highly significant) (Table 3). Supplementary Table S3 details the outcome of the statistical tests performed.

TSG hypermethylation in relation to *SDHB* mutation

The heat map in Fig. 3 illustrates the patterns of TSG hypermethylation in cases with different forms of heritable disease. Altogether, 32 primary tumors and two metastases from the combined Series A+B were from patients with a hereditary form of the disease based on (familial) syndromic presentation and/or identified germ-line mutations. The 11 *SDHB*-positive tumors from ten cases exhibited promoter hypermethylation for two or more of the investigated genes concerning MetI or the maximum for at least one individual CpG. Specifically, increased methylation was observed in 11/11 tumors for *P16*, 9/11 for *DCR2*, 7/11 for *RASSF1A*, 6/11 for *CDH1*, 5/11 for *RARB*, and 4/11 for *NORE1A* (Fig. 3). For *P16*, *DCR2*, and *RASSF1A*, the hypermethylation was, with few exceptions, pronounced, with MetI above cutoff. By contrast, tumors from patients associated with *RET*/MEN2 ($n=12$), NF1 ($n=7$), or *VHL*/VHL ($n=3$) exhibited increased methylation for individual CpGs in nine instances only, and MetI did not exceed background level. Taken together, the combined observations in Series A+B strongly support an association between *SDHB* mutation and TSG hypermethylation, with *P16* being the most frequently involved.

Order of genetic events and TSG hypermethylation in tumor development

To evaluate the relationship between occurrence of genetic and epigenetic events in the tumor development,

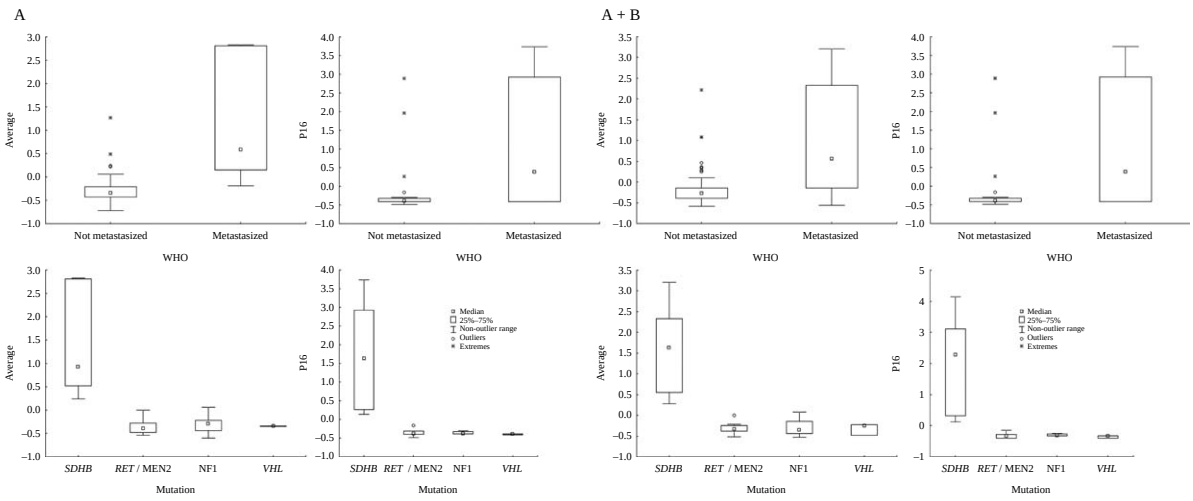

Figure 2
Boxplots illustrate the significant associations in Series A and A+B between increased mean *Z*-scores and metastatic disease and *SDHB* mutation, as well as between high *P16* *Z*-score and *SDHB* mutation.

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

Figure 3

Association between *SDHB* mutation, tumor suppressor gene hypermethylation, and metastasis. Methylation densities are illustrated for individual tumors in Series A + B from patients with predisposing mutations and/or syndromic disease related to *SDHB*, *RET*/MEN2, NF1, or *VHL*/VHL. Tumors with MetI levels above cutoff are indicated by dark blue boxes, above cutoff for one or more CpG sites by light blue boxes, and below cutoff levels by green boxes. Cutoff levels for individual genes are >30% for *DCR2* and *RASSF1A* and >10% for *CDH1*, *NORE1A*, *P16*, and *RARB*. Cases with metastasis are indicated to the right. *Metastasis from the tumor in the row above.

matched samples of normal and tumor tissues were compared in five cases from Series B (Fig. 4; Supplementary Table S1). All five primary tumors and two metastases from the five patients have previously been reported to carry increased promoter MetI in two to five of the investigated genes *DCR2*, *CDH1*, *NORE1A*, *P16*, *RARB*, or *RASSF1A* (Geli *et al*. 2007, 2008, Kiss *et al*. 2008). In four of the five cases, three or more TSG promoters showed increased MetI in agreement with a CIMP phenotype. By contrast, pyrosequencing of the same promoters in constitutional DNA revealed only very low levels of CpG methylation in all five cases (Supplementary Table S1).

Four of the five cases carried a constitutional inactivating mutation of the *SDHB* gene that was present in normal, tumor, and metastasis analyzed. In mutated cases, double peaks of mutated and wild-type sequences were observed in constitutional DNA, in agreement with heterozygous state of the mutations (Supplementary Figure S1, see section on supplementary data given at the end of this article). The occurrence of genetic and epigenetic events from constitutional tissue to primary tumor and metastasis is outlined in Fig. 4 for the five cases. Hence, *SDHB* mutations were present constitutionally, while TSG hypermethylation and CIMP were acquired events first observed in primary tumors together with loss of chromosomal region 1p encompassing the *SDHB* locus, and subsequently retained to metastatic tissue.

Global methylation of *LINE1* repeat elements

MetI levels for *LINE1* repeat elements varied from 46 to 80% in the 39 primary tumors in Series A, and the values at the individual CpGs ranged from 37 to 83% (Table 2). The two metastases exhibited MetI of 71 and 76% (range 70–79%). These results overlap with those in normal adrenal references, where MetI at 68–71% and individual CpGs methylation within the range 65–76% are observed. *LINE1 Z*-scores differed significantly between the genders (higher mean value in males).

Discussion

We here report significantly higher mean TSG methylation levels in paragangliomas, metastasizing tumors,

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

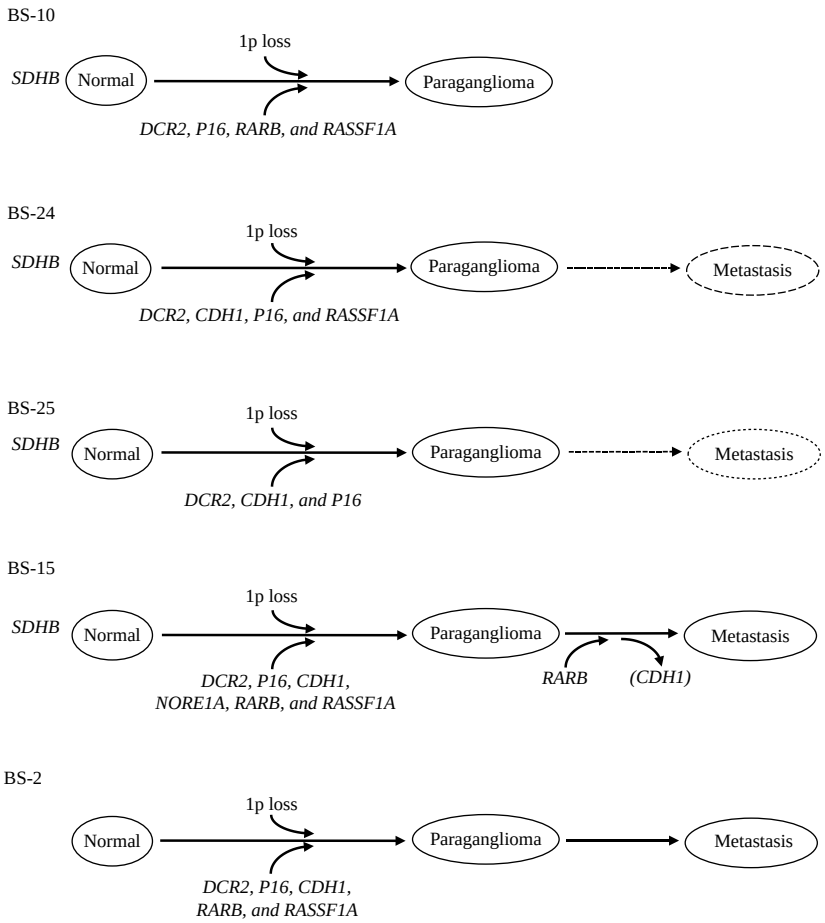

Figure 4

Schematic illustrations of genetic and epigenetic events detected in normal tissue, primary tumors, and metastasis from tumor Series B. Genetic events concerning constitutional *SDHB* mutations and somatic loss of 1p in primary tumors are illustrated above the flow charts. Detection of promoter hypermethylation – reflected in increased MetI – and methylation above the cutoff for one or more CpG only (in parenthesis) are indicated below for the TSG promoter concerned (see Supplementary Table S1 for exact values). Samples assayed are shown in bold and by solid lines, while metastases not assayed are indicated by dotted lines.

SDHB-mutated tumors, and cases with poor survival (Fig. 2; Table 3). Taken together, these findings in the novel tumor Series A powerfully substantiate similar observations from Series B (Geli *et al*. 2008).

We have previously defined CIMP as mean CpG hypermethylation above the normal range cutoff in three or more TSG promoters. To herein provide a more detailed assessment of the epigenetic events in these tumors, we have also considered methylation at individual CpGs (Fig. 1; Table 2). *Z*-scores denoting individual TSG methylation levels, as well as mean *Z*-scores for all TSGs in each case, were compared with clinical and genetic features (Table 3).

Indeed, individual TSG hypermethylation was salient in paragangliomas, being especially prominent in tumors classified as malignant. In the combined Series A+B, 7/11 metastasized paragangliomas exhibited MetI above cutoff in two or more of the assessed TSG promoters (Fig. 5). Further, 4/12 paragangliomas without metastases carried *SDHB* mutations – strongly linked to malignant behavior and metastatic potential (Boedeker 2011) – three of which also exhibited MetI above cutoff for two or more TSGs (Fig. 5). In contrast, TSG hypermethylation proved infrequent in pheochromocytomas; 5/54 cases featured MetI above cutoff in a single TSG each (Fig. 5). The most frequently hypermethylated genes were *DCR2* (eight tumors), *P16* (11 tumors (AG-6 exhibited elevated MetI, but was nonetheless. Five percentage points too low to reach cutoff)), and *RASSF1A* (nine tumors), indicating that epigenetic modifications

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

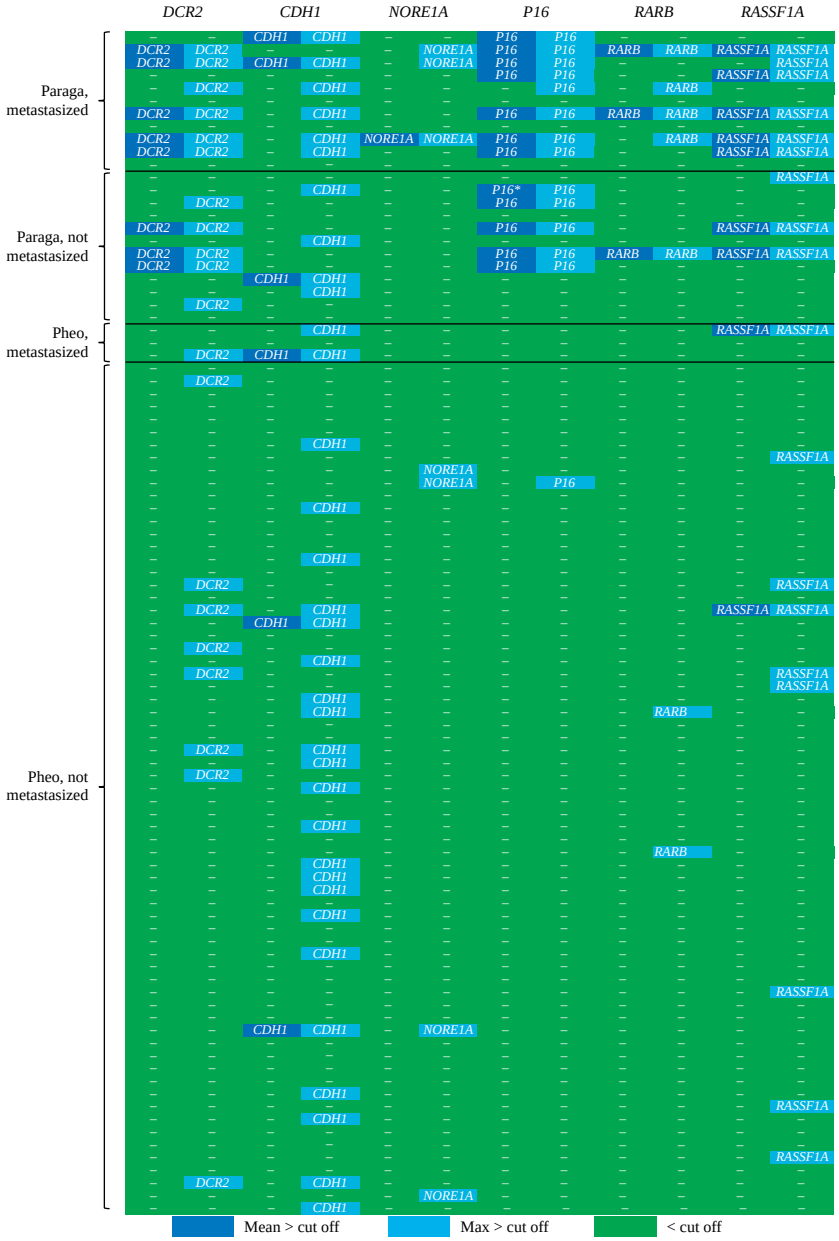

Figure 5

Comparison of promoter hypermethylation at individual tumor suppressor gene promoters in paraganglioma and pheochromocytoma with and without metastasis from tumor combined Series A+B. Methylation levels are indicated as dark blue boxes for MetI above cutoff, light blue boxes refer to one or more CpG sites above cutoff, and green boxes denote promoters without detected hypermethylation. Cutoff levels for individual genes were applied at >30% (*DCR2* and *RASSF1A*) or >10% (*CDH1*, *NORE1A*, *P16*, and *RARB*). *AG-6 displays elevated *P16* MetI just under cutoff.

of these genes is an important facet of malignancy in paragangliomas.

We found a very strong association between *SDHB* mutation and hypermethylation of several TSGs in the novel Series A (Fig. 2; Table 3), in agreement with our previous reports of an association to *P16* hypermethylation (Kiss *et al.* 2008). The *Z*-score box plots in Fig. 2, grouped by syndrome/mutation, further highlight the over-representation of TSG CpG methylation coinciding with *SDHB* mutation compared with other genetic variants. *P16* hypermethylation was most frequently seen with and was unequivocally associated

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

with *SDHB* mutation (Fig. 2; Table 2). Furthermore, for the paraganglioma AG-6 with *SDHB* mutation, the MetI for *P16* was elevated and close to the nominal 10% cutoff value, and hypermethylation above cutoff was observed for individual CpGs. Similarly, the *SDHB*-mutated paraganglioma lymph node metastasis AS-21 exhibited a MetI of 8% (range 4–12%) for *P16*. This observation is perhaps indicative of a developing phenotype or a result of tumor heterogeneity. By contrast, MetI levels for *P16* were very low (1 or 2%) in tumors without detectable *SDHB* mutation. Figure 3 further illustrates that in the combined Series A+B, concerted epigenetic events occur in relation to *SDHB* mutation but are not observed in association with other predisposing syndromes. Survival analyses of combined Series A+B demonstrated significantly shorter survival in patients with primary tumors that displayed *P16* MetI >10% compared with those with MetI below cutoff (Fig. 6).

In Series A as well as in combined Series A+B, hypermethylation of TSGs was found in association with paraganglioma tumor type, development of metastases, and mutation of the predisposing gene *SDHB*. While metastasis and *SDHB* mutations are known to be correlated, the question arises whether hypermethylation also occurs in *SDHB* wild-type metastatic tumors. *SDHB* mutations were not identified in 6 of the 14 metastatic tumors in this study. Among these, only two paragangliomas had hypermethylation. Significant methylation in *RASSF1A* alone was detected when analyzing TSG methylation in all metastatic tumors without apparent *SDHB* involvement ($P \geq 0.02$). Hence, increased TSG methylation does not appear to be frequently involved in sporadic, malignant cases or in conjunction with other predisposing mutations. However, it is presently unknown whether metastatic tumors with mutations other than *SDHB* harbor TSG methylation. We see no such involvement in benign tumors with known mutations other than *SDHB* (Fig. 3).

SDHB mutations are known to be frequently associated with malignant forms of paraganglioma (Ricketts *et al.* 2010), raising questions about causes and consequences in relation to these abnormalities and their clinical effects. In four cases with constitutional *SDHB* mutations in Series B, TSG hypermethylation was absent in constitutional DNA – indicating that the TSG hypermethylation is tumor-specific – and first observed in primary tumors in conjunction with loss of 1p encompassing the *SDHB* gene locus (Fig. 4). Furthermore, acquired TSG hypermethylation was observed in four malignant primary paragangliomas before the development of metastasis and one case where metastasis had not developed before surgery

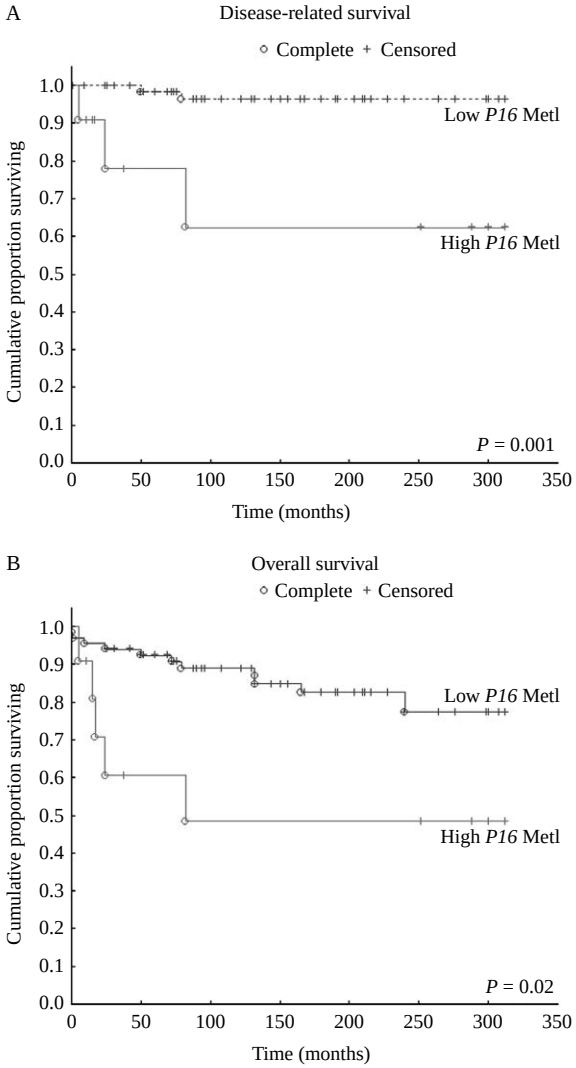

Figure 6
Kaplan–Meyer plots illustrating the significant association between short disease-related (A) and overall (B) survival and hypermethylation of *P16* based on data from combined Series A+B.

(BS-10; Fig. 4). Importantly, these findings implicate that TSG hypermethylation is not a secondary consequence of a malignant tumor state. Furthermore, they would imply that heterozygous *SDHB* inactivating mutations do not confer detectable TSG hypermethylation at the constitutional level, at least not for the tissues/genes assessed here. However, it is a theoretical possibility that heterozygous *SDHB* inactivation could lead to TSG hypermethylation in cancer progenitor cells of the target tissue and subsequently be selected for at tumor transformation.

The data presented here strongly indicate that *SDHB* inactivation and TSG CpG hypermethylation are

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0267

Published by Bioscientifica Ltd.

associated; an attractive possibility being that *SDHB* inactivation in fact causes TSG hypermethylation. To our knowledge, this would represent the first instance where a known hereditary cancer syndrome (familial paraganglioma syndrome type 4) is linked to an epigenetic phenotype. The link between SDH loss and epigenetic remodeling represented by histone modifications was recently shown (Smith *et al*. 2007, Cervera *et al*. 2009). Importantly, the breakdown of the mitochondrial respiratory chain by loss of SDH function leads to accumulation of succinate, which causes inhibition of α-ketoglutarate-dependent enzymes (that normally produce succinate as a byproduct; Lee *et al*. 2005, Selak *et al*. 2005). Among them are the jumonji-domain histone demethylases (Smith *et al*. 2007) that regulate histone H3 and H4 lysine and arginine methylation (Agger *et al*. 2008, Cervera *et al*. 2009). This would overture a general inhibition of histone demethylase activity (Cervera *et al*. 2009), likely affecting chromatin on a global level. It is plausible that these histone modifications and the epigenetic perturbances observed in the current project have a joint causality. However, we observe a very specific pattern of promoter CpG methylation in our tumor panel, while global methylation patterns analogized by *LINE1* remain heterogeneous regardless of *SDHB* status. This specific methylative inactivation of apoptotic and antiproliferative genes might instead mirror a physiological attempt to counter the state of pseudo-hypoxia, induced by SDH dysfunction and described in Pollard *et al*. (2005) and Cervera *et al*. (2008). Indeed, recent immunohistochemical studies have shown that tumors from patients with *SDHB*, *SDHC*, and *SDHD* mutations lack SDHB immunoreactivity and that SDH activity is abolished in *SDHB*- and *SDHD*-mutated cases but not in connection with *SDHC* (van Nederveen *et al*. 2009). These observations contrast those in tumors from patients with predisposing mutations in other genes. Based on the results, the authors recommended that screening for SDH gene mutations should be carried out in cases with negative SDHB immunohistochemistry (van Nederveen *et al*. 2009). Another implication from these interesting findings is that the increased risk of malignancy associated with SDHB would not be a direct result of SDHB protein loss but relate to other molecular abnormalities perhaps occurring at the transcriptional level.

Recognition of constitutional mutation carriers among pheochromocytoma and paraganglioma patients is of clinical importance but in principle requires extensive mutation screenings in a large group of patients – as several genes are involved, and hereditary predisposition is often present in spite of a negative family history. The findings presented here suggest that methylation quantification for *P16* promoter CpGs could be a valuable clinical tool in the assessment of paragangliomas and that cases with *P16* hypermethylation should be genetically screened for *SDHB* mutations.

In summary, our results associated mutation of the *SDHB* gene to alterations in TSG methylation in paragangliomas. We here propose that epigenetic inactivation of TSGs is an important component in familial paraganglioma syndrome and suggest inquiries into the use of demethylating agents as a means to combat malignant paragangliomas. Future analyses should also encompass the assessment of gene-specific methylation in *SDHB*-deficient paragangliomas using methylation arrays. We further propose the use of *P16* methylation assessment as an additive tool in identification of patients for *SDHB* mutation screenings.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0267.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
The study was financially supported by grants from Swedish Cancer Society, Swedish Research Council, Göran Gustafsson Foundation for Research in Natural Sciences and Medicine, Gustav V Jubilee Foundation, Karolinska Institutet, and Stockholm County Council.

Acknowledgements
Dedicated to the memory of Håkan Ahlman, 1947–2012. The authors wish to thank Stefano Caramuta for expert assistance with survival analyses.

References

Agger K, Christensen J, Cloos PAC & Helin K 2008 The emerging functions of histone demethylases. *Current Opinion in Genetics & Development* **18** 159–168. (doi:10.1016/j.gde.2007.12.003)

Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, Mey A, Taschner PEM, Rubinstein WS & Myers EN 2000 Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* **287** 848. (doi:10.1126/science.287.5454.848)

Benn DE, Croxson MS, Tucker K, Bambach CP, Richardson AL, Delbridge L, Pullan PT, Hammond J, Marsh DJ & Robinson BG 2003 Novel succinate dehydrogenase subunit B (SDHB) mutations in familial phaeochromocytomas and paragangliomas, but an absence of somatic SDHB mutations in sporadic phaeochromocytomas. *Oncogene* **22** 1358–1364. (doi:10.1038/sj.onc.1206300)

Published by Bioscientifica Ltd.

Boedeker C 2011 Paragangliomas and paraganglioma syndromes. *Laryngo-Rhino-Otologie* **90** S56–S82. (doi:10.1055/s-0030-1270447)

Burnichon N, Brière JJ, Libé R, Vescovo L, Rivière J, Tissier F, Jouanno E, Jeunemaitre X, Bénit P & Tzagoloff A 2010 SDHA is a tumor suppressor gene causing paraganglioma. *Human Molecular Genetics* **19** 3011. (doi:10.1093/hmg/ddq206)

Cascon A, Ruiz-Llorente S, Fraga M, Leton R, Telleria D, Sastre J, Diez JJ, Diaz-Guerra GM, Perez JAD & Benitez J 2004 Genetic and epigenetic profile of sporadic pheochromocytomas. *Journal of Medical Genetics* **41** e30–e30. (doi:10.1136/jmg.2003.012658)

Castellano M, Mori L, Giacche M, Agliozzo E, Tosini R, Panarotto A, Cappelli C, Mulatero P, Cumetti D & Veglio F 2006 Genetic mutation screening in an Italian cohort of nonsyndromic pheochromocytoma/paraganglioma patients. *Annals of the New York Academy of Sciences* **1073** 156–165. (doi:10.1196/annals.1353.016)

Cervera AM, Apostolova N, Crespo FL, Mata M & McCreath KJ 2008 Cells silenced for SDHB expression display characteristic features of the tumor phenotype. *Cancer Research* **68** 4058. (doi:10.1158/0008-5472.CAN-07-5580)

Cervera AM, Bayley JP, Devilee P & McCreath KJ 2009 Inhibition of succinate dehydrogenase dysregulates histone modifications in mammalian cells. *Molecular Cancer* **8** 89. (doi:10.1186/1476-4598-8-89)

Comino-Méndez I, Gracia-Aznárez FJ, Schiavi F, Landa I, Leandro-García LJ, Letón R, Honrado E, Ramos-Medina R, Caronia D & Pita G 2011 Exome sequencing identifies MAX mutations as a cause of hereditary pheochromocytoma. *Nature Genetics* **43** 663–667. (doi:10.1038/ng.861)

DeLellis RA 2004 Tumors of the adrenal gland. In *World Health Organization Classification of Tumors. Pathology & Genetics*, pp136–166. Eds RA DeLellis, RV Lloyd, PU Heitz & C Eng. Lyon, France: IARC Press.

Edström Elder E, Hjelm Skog AL, Höög A & Hamberger B 2003 The management of benign and malignant pheochromocytoma and abdominal paraganglioma. *European Journal of Surgical Oncology* **29** 278–283. (doi:10.1053/ejso.2002.1413)

Elder EE, Elder G & Larsson C 2005 Pheochromocytoma and functional paraganglioma syndrome: no longer the 10% tumor. *Journal of Surgical Oncology* **89** 193–201. (doi:10.1002/jso.20177)

Geli J, Kiss N, Lanner F, Foukakis T, Natalishvili N, Larsson O, Kogner P, Höög A, Clark GJ & Ekström TJ 2007 The Ras effectors NORE1A and RASSF1A are frequently inactivated in pheochromocytoma and abdominal paraganglioma. *Endocrine-Related Cancer* **14** 125–134. (doi:10.1677/ERC-06-0031)

Geli J, Kiss N, Karimi M, Lee JJ, Bäckdahl M, Ekström TJ & Larsson C 2008 Global and regional CpG methylation in pheochromocytomas and abdominal paragangliomas: association to malignant behavior. *Clinical Cancer Research* **14** 2551–2559. (doi:10.1158/1078-0432.CCR-07-1867)

Hao HX, Khalimonchuk O, Schraders M, Dephoure N, Bayley JP, Kunst H, Devilee P, Cremers CW, Schiffman JD & Bentz BG 2009 SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. *Science* **325** 1139–1142. (doi:10.1126/science.1175689)

Khorram-Manesh A, Ahlman H, Nilsson O, Friberg P, Oden A, Stenström G, Hansson G, Stenquist O, Wängberg B & Tisell LE 2005 Long-term outcome of a large series of patients surgically treated for pheochromocytoma. *Journal of Internal Medicine* **258** 55–66. (doi:10.1111/j.1365-2796.2005.01504.x)

Kiss N, Geli J, Lundberg F, Avci C, Velazquez-Fernandez D, Hashemi J, Weber G, Höög A, Ekström T & Bäckdahl M 2008 Methylation of the p16INK4A promoter is associated with malignant behavior in abdominal extra-adrenal paragangliomas but not pheochromocytomas. *Endocrine-Related Cancer* **15** 609–621. (doi:10.1677/ERC-07-0285)

Kiss NB, Kogner P, Johnsen JI, Martinsson T, Larsson C & Geli J 2012 Quantitative global and gene-specific promoter methylation in relation to biological properties of neuroblastomas. *BMC Medical Genetics* **13** 83. (doi:10.1186/1471-2350-13-83)

Lee S, Nakamura E, Yang H, Wei W, Linggi MS, Sajan MP, Farese RV, Freeman RS, Carter BD & Kaelin WG, Jr 2005 Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. *Cancer Cell* **8** 155–167. (doi:10.1016/j.ccr.2005.06.015)

Lin Y, Diccianni M, Kim Y, Lin H, Lee C, Lin R, Joo S, Li J, Chuang T & Yang A 2007 Human p16γ, a novel transcriptional variant of p16INK4A, coexpresses with p16INK4A in cancer cells and inhibits cell-cycle progression. *Oncogene* **26** 7017–7027. (doi:10.1038/sj.onc.1210507)

Margetts C, Astuti D, Gentle D, Cooper W, Cascon A, Catchpoole D, Robledo M, Neumann H, Latif F & Maher E 2005 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. *Endocrine-Related Cancer* **12** 161–172. (doi:10.1677/erc.1.00865)

Muscarella P, Bloomston M, Brewer AR, Mahajan A, Frankel WL, Ellison EC, Farrar WB, Weghorst CM & Li J 2008 Expression of the p16INK4A/Cdkn2a gene is prevalently downregulated in human pheochromocytoma tumor specimens. *Gene Expression* **14** 207–216. (doi:10.3727/105221608786883825)

Muth A, Abel F, Jansson S, Nilsson O, Ahlman H & Wängberg B 2012 Prevalence of germline mutations in patients with pheochromocytoma or abdominal paraganglioma and sporadic presentation: a population-based study in western Sweden. *World Journal of Surgery* **36** 1389–1394. (doi:10.1007/s00268-012-1430-6)

van Nederveen FH, Gaal J, Favier J, Korpershoek E, Oldenburg RA, de Bruyn EMCA, Sleddens HF, Derkx P, Rivière J & Dannenberg H 2009 An immunohistochemical procedure to detect patients with paraganglioma and phaeochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis. *Lancet Oncology* **10** 764–771. (doi:10.1016/S1470-2045(09)70164-0)

Neumann HPH, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C & Zerres K 2002 Germ-line mutations in nonsyndromic pheochromocytoma. *New England Journal of Medicine* **346** 1459–1466. (doi:10.1056/NEJMoa020152)

Pollard P, Briere J, Alam N, Barwell J, Barclay E, Wortham N, Hunt T, Mitchell M, Olpin S & Moat SJ 2005 Accumulation of Krebs cycle intermediates and over-expression of HIF1α in tumours which result from germline FH and SDH mutations. *Human Molecular Genetics* **14** 2231. (doi:10.1093/hmg/ddi227)

Qin Y, Yao L, King EE, Buddavarapu K, Lenci RE, Chocron ES, Lechleiter JD, Sass M, Aronin N & Schiavi F 2010 Germline mutations in TMEM127 confer susceptibility to pheochromocytoma. *Nature Genetics* **42** 229–233. (doi:10.1038/ng.533)

Ricketts CJ, Forman JR, Rattenberry E, Bradshaw N, Lalloo F, Izatt L, Cole TR, Armstrong R, Kumar V & Morrison PJ 2010 Tumor risks and genotype–phenotype–proteotype analysis in 358 patients with germline mutations in SDHB and SDHD. *Human Mutation* **31** 41–51. (doi:10.1002/humu.21136)

Sandgren J, Andersson R, Rada-Iglesias A, Enroth S, Åkerström G, Dumanski JP, Komorowski J, Westin G & Wadelius C 2010*a* Integrative epigenomic and genomic analysis of malignant pheochromocytoma. *Experimental & Molecular Medicine* **42** 484. (doi:10.3858/emm.2010.42.7.050)

Sandgren J, de Ståhl TD, Andersson R, Menzel U, Piotrowski A, Nord H, Bäckdahl M, Kiss NB, Brauckhoff M & Komorowski J 2010*b* Recurrent genomic alterations in benign and malignant pheochromocytomas and paragangliomas revealed by whole-genome array comparative genomic hybridization analysis. *Endocrine-Related Cancer* **17** 561–579. (doi:10.1677/ERC-09-0310)

Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB & Gottlieb E 2005 Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-[α] prolyl hydroxylase. *Cancer Cell* **7** 77–85. (doi:10.1016/j.ccr.2004.11.022)

Smith EH, Janknecht R & Maher LJ 2007 Succinate inhibition of α-ketoglutarate-dependent enzymes in a yeast model of

Published by Bioscientifica Ltd.

paraganglioma. *Human Molecular Genetics* **16** 3136. (doi:10.1093/hmg/ddm275)

Tischler AS 2008 Pheochromocytoma and extra-adrenal paraganglioma: updates. *Archives of Pathology & Laboratory Medicine* **132** 1272–1284. (doi:10.1043/1543-2165(2008)132[1272:PAEPU]2.0.CO;2)

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB & Issa JPJ 1999 CpG island methylator phenotype in colorectal cancer. *PNAS* **96** 8681. (doi:10.1073/pnas.96.15.8681)

Wängberg B, Muth A, Khorram-Manesh A, Jansson S, Nilsson O, Forssell-Aronsson E, Tisell L & Ahlman H 2006 Malignant pheochromocytoma in a population-based study. *Annals of the New York Academy of Sciences* **1073** 512–516. (doi:10.1196/annals.1353.054)

Yao L, Schiavi F, Cascon A, Qin Y, Inglada-Pérez L, King EE, Toledo RA, Ercolino T, Rapizzi E & Ricketts CJ 2010 Spectrum and prevalence of FP/TMEM127 gene mutations in pheochromocytomas and paragangliomas. *Journal of the American Medical Association* **304** 2611–2619. (doi:10.1001/jama.2010.1830)

You MJ, Castrillon DH, Bastian BC, O'Hagan RC, Bosenberg MW, Parsons R, Chin L & DePinho RA 2002 Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. *PNAS* **99** 1455. (doi:10.1073/pnas.022632099)

Received in final form 7 November 2012
Accepted 15 November 2012
Made available online as an Accepted Preprint
15 November 2012

Published by Bioscientifica Ltd.