

Global and Regional CpG Methylation in Pheochromocytomas and Abdominal Paragangliomas: Association to Malignant Behavior

Janos Geli,¹ Nimrod Kiss,¹ Mohsen Karimi,² Jia-Jing Lee,¹ Martin Bäckdahl,¹ Tomas J. Ekström,² and Catharina Larsson¹

Abstract **Purpose:** This study aims to quantitatively assess promoter and global methylation changes in pheochromocytomas and abdominal paragangliomas and its relation to tumor phenotypes. **Experimental Design:** A panel of 53 primary tumors (42 benign, 11 malignant) was analyzed by quantitative bisulfite pyrosequencing. Based on methylation levels in the tumor suppressor genes, *p16^{INK4A}*, *CDH1*, *DCR2*, *RARB*, *RASSF1A*, *NORE1A*, *TP73*, *APC*, *DAPK1*, *p14^{ARF}*, and *PTEN*, a CpG island methylator phenotype (CIMP) was defined as concerted hypermethylation in three or more genes. Mean Z scores for the hypermethylated promoters were calculated to characterize overall promoter methylation. Global DNA methylation was quantified for *LINE-1* promoter sequences and by using luminescent methylation analysis. **Results:** Five primary tumors (9.4%) exhibited a CIMP phenotype, four of which were malignant paragangliomas. CIMP was significantly associated with malignant behavior ($P = 0.005$) and younger age at presentation ($P < 0.007$) but did not result from *BRAF* V600E mutation. Global hypomethylation of *LINE-1* elements was observed in tumors compared with normal adrenal samples ($P < 0.02$). **Conclusion:** We here describe the identification of CIMP in abdominal paragangliomas and a strong association of this phenotype with malignant behavior, as well as young age at presentation. The findings raise a prospective for potential benefits of epigenetically acting drugs for a subgroup of young abdominal paraganglioma patients with adverse prognosis.

DNA methylation of CpG-rich promoters is a common mechanism for epigenetic gene silencing and is linked to physiologic processes, such as the inactivation of X chromosome, genetic imprinting, and aging (1). In cancer, hypermethylation of promoter regions serves as an alternative mechanism of tumor suppressor inactivation (2) in addition to classic structural mutations. The term CpG island methylator phenotype (CIMP) refers to the concordant occurrence of consistently increased methylation in multiple genes. This putative phenotype was first described in colon cancer (3) and subsequently in other tumors, such as those of the gastrointestinal

tract (4–7), nervous (8, 9), and hematopoietic systems (10, 11). Several studies associated CIMP with specific somatic genetic alterations, such as *KRAS* or *BRAF* mutations in colorectal cancer (12, 13) and *MYCN* amplification in neuroblastomas (8, 14). Moreover, some observations also suggested generally increased cancer prevalence among first-grade relatives to patients diagnosed with CIMP-positive colonic lesions (15, 16). An eminent aspect for the clinician is whether or not CIMP translates into differential prognosis. In several but not all (17) neoplasias, CIMP is identified as a strong prognostic factor predicting unfavorable outcome (4, 8, 11, 14, 18).

Besides CpG island hypermethylation global hypomethylation is also an epigenetic alteration frequently observed in various cancers (19, 20). It remains to be further elucidated whether these seemingly opposing trends have any mechanistic connection. At present, little is known about alterations of genome-wide methylation in the context of neuroendocrine tumors.

Pheochromocytomas and abdominal paragangliomas (the latter are in here called paraganglioma and in the literature often as extraadrenal pheochromocytoma) are chromaffin cell-derived tumors of the sympathetic nervous system located in the adrenal medulla or the sympathetic paraganglia, respectively (21). Recent advances have shown that genetic predisposition, by constitutional mutations in, e.g., *RET*, *VHL*, *SDHD*, or *SDHB*, is far more prevalent in these tumors than previously assumed and may account for approximately a quarter of cases (22).

Authors' Affiliations: Departments of ¹Molecular Medicine and Surgery and ²Clinical Neuroscience, Karolinska Institutet, Karolinska University Hospital Solna, Stockholm, Sweden

Received 7/29/07; revised 12/20/07; accepted 1/29/08.

Grant support: Swedish Cancer Society, Göran Gustafsson Foundation for Research in Natural Sciences and Medicine, Gustav V Jubilee Foundation, Stockholm Cancer Society, Karolinska Institutet, and Stockholm County Council.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Requests for reprints: Janos Geli, Department of Molecular Medicine and Surgery, Karolinska University Hospital Solna, CMM L8:01, SE-171 76 Stockholm, Sweden. Phone: 46-8-51773616; Fax: 46-8-51776180; E-mail: Janos.Geli@ki.se.

©2008 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-07-1867

A number of recent studies have attempted to further delineate the pattern of promoter methylation³ (23–27) in pheochromocytomas/paragangliomas. One of the genes found to show consistent methylation across various reports is *RASSF1A*. Most of these studies used nonquantitative methods (24, 25, 27) that are highly sensitive but with the drawback of being unable to distinguish tumors with substantial methylation from those with biologically insignificant levels (28, 29). This may make the interpretation of methylation data difficult, especially when concordant methylation of multiple genes is analyzed to correlate it to clinicopathologic features. Quantitative detection methods of DNA methylation provide an attractive approach and is becoming increasingly used in methylation studies (17, 26, 30–33). Recent experimental data suggest that CIMP is best assessed by quantitative DNA methylation analysis as shown for colorectal cancer, thus far, the most extensively characterized tumor for this molecular phenotype (34).

In the present study, we sought to explore the occurrence and relevance of CIMP in pheochromocytomas and paragangliomas. To this aim, promoter methylation of a set of tumor suppressor genes, as well as global methylation, was quantitatively determined and evaluated in relation to clinical characteristics.

Materials and Methods

Tumors and reference samples. The study includes a panel of 55 tumors: 43 primary pheochromocytomas (37 benign, 6 malignant), 10 primary paragangliomas (5 benign, 5 malignant), and 2 paraganglioma distant metastases (Table 1). Cases 1 and 2, as well as cases 3 and 4, are matched primary tumors and metastases. Six tumors are from patients with a known familial form of the disease (syndromic presentation), including multiple endocrine neoplasia type 2A (cases 25, 30, and 44), neurofibromatosis type 1 (cases 26 and 45), and von Hippel-Lindau disease (case 36). Patients were operated at the Endocrine Surgical Unit at the Karolinska University Hospital Solna, and clinical characteristics have been previously described (35) and published (26). All samples included in the study had been snap-frozen in liquid nitrogen after surgery, stored at -70°C, and verified to contain a high purity (>70%) of tumor cells by histopathologic examination. Tumor classification was according to the criteria of the U.S. Armed Forces Institute of Pathology, i.e., the diagnosis of malignancy required extensive local invasion and/or distant metastasis (21, 36). Informed consent was obtained from each patient, and study of the tissue material was approved by the institutional ethical review board.

Normal controls were constituted of adrenal medullary DNA (Norm 1-4) derived from four Caucasian individuals (ages, 50-76 y) who had no medical history of neoplastic disease and had died due to noncancer causes (Clinomics Biosciences, Inc.).

DNA extraction and bisulfite treatment. High molecular weight DNA was extracted using a method based on proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. For luminescent methylation analysis (LUMA), DNA was extracted using GeneElute Mammalian Genomic DNA Miniprep kit (Sigma Aldrich). Sodium bisulfite modification of 2 µg DNA was done with the EZ DNA methylation kit (Zymo Research Corporation) following the manufacturer's protocol.

Quantitative evaluation of promoter methylation by bisulfite pyrosequencing analysis. Pyrosequencing-based methylation analysis was done to interrogate methylation density at the promoters of *CDH1*, *DCR2*, *RARB*, *TP73*, *APC*, *DAPK1*, and *PTEN*. Results from similar analyses of *p16^{INK4A}*, *p14^{ARF}*, *RASSF1A*, and *NORE1A* have been reported elsewhere (26).³ The PCR assays focus on a segment of the promoter CpG islands of the investigated genes, and primers target CpG-free regions to ensure methylation-independent amplification. Primer sequences are available at the PyroMark Assay Database (Biotage AB). HotStar Taq polymerase and HotStar Taqs Master Mix kit (Qiagen Ltd.) was used to amplify 1.5 µL bisulfite-treated DNA. Cycling conditions were 95°C 15 min, 45 × (95°C 20 s, 50-55°C 20 s, 72°C 20 s), 72°C 10 min, 4°C. Quality of the PCR product was ascertained by electrophoresis in 3% agarose gel with ethidium bromide staining.

Pyrosequencing was performed at Biotage using the PSQ HS96 system (Biotage AB) including PyroGold reagents. During pyrosequencing reactions, the pyrosequencing primers interrogate a stretch of DNA containing 4 to 19 target CpGs (Table 2); inclusion of CpG sites in the primer target sequence is again avoided. The resulting pyrograms were further analyzed for quantification of methylation density using the Pyro Q-CpG software (Biotage AB). Due to the methylation-dependent sequence alteration upon bisulfite treatment of DNA, the ratio of C/T peaks at each individual CpG target site accurately reflects the proportion of methylated to nonmethylated alleles, i.e., the methylation density is expressed as percentage of the total. A non-CpG C within the assayed sequences serves as internal control for bisulfite conversion efficiency. In all runs, *Sss1*-treated and untreated normal lymphocyte DNA with and without bisulfite treatment was included as controls.

Measurement of global methylation. Global methylation was determined by two distinct methods. CpG methylation of *LINE-1* promoter elements (37) was determined by quantitative bisulfite pyrosequencing using the PyroMark *LINE-1* assay (Biotage AB). LUMA was used to quantify global methylation levels by assessing methylation of CCGG restriction sites across the genome, according to previously described methodology (38). In short, genomic DNA was separately cleaved with *HpaII* + *EcoRI* and *MspI* + *EcoRI*, and the degree of cleavage was subsequently quantified by pyrosequencing. The calculated ratio *HpaII/MspI* (ranging from 0 to 1) represents the degree of methylation.

Screening for the BRAF mutation V600E. All samples were screened for the common *BRAF* mutation GTG→GAG at nucleotide 1799 in exon 15, which leads to a missense mutation V600E. Exon 15 was amplified by PCR and sequenced using BigDye Terminator v3.1 (Applied Biosystems) and an ABI 3700 sequencer (Applied Biosystems). Thyroid carcinoma cells served as positive controls for the mutated status (39, 40).

Statistical analysis of bisulfite pyrosequencing data. For all statistical calculations, STATISTICA data analysis software system version 7 was used (Statsoft, Inc.), and *P* values of ≤0.05 were considered significant. Statistical analyses only comprised data from the 53 primary tumor samples, whereas the two metastases (cases 2 and 4) were excluded from the calculations. This was done taking into consideration the fundamental biological differences between primary and metastatic lesions, and the fact that the primary lesion from the same patients was already included in the data set. For each sample and promoter, an average methylation density was calculated based on the methylation densities at the individual CpGs within the assayed promoter sequence. Tumors demonstrating mean methylation density above the range observed in normal tissue in three or more promoters were classified as CIMP (Tables 2 and 3). To enable the assessment of concerted methylation at multiple promoters as a continuous variable, *Z* score analysis was used (Table 2; ref. 41). A *Z* score for each of the following seven gene promoters (*DCR2*, *RASSF1A*, *NORE1A*, *p16^{INK4A}*, *RARB*, *CDH1*, *APC*) was computed using the given formula: (mean of CpG methylation density of the assessed promoter for each sample - mean of methylation density for the tumor panel) / SD of methylation density. For each case, a mean *Z* score was calculated incorporating the promoter specific *Z* scores and used subsequently as a single measure

³ NB Kiss, J Geli, F Lundberg, C Avci, D Velazquez-Fernandez, J Hashemi, G Weber, A Höög, TJ Ekström, M Bäckdahl, C Larsson. Methylation of the *p16^{INK4A}* promoter is associated with malignant behavior in abdominal extra-adrenal paragangliomas but not pheochromocytomas. Endocrine-Related Cancer; in press.

characterizing methylation density (Table 2). Thus, a mean Z score of >0 reflects an overall promoter methylation density higher than the population average. The following features were assessed: CIMP versus non-CIMP status, global methylation by *LINE-1* and LUMA, malignancy, histopathology (i.e., pheochromocytoma or paraganglioma),

sex, syndromic versus nonsyndromic presentation, age at diagnosis, and tumor size. Comparisons of categorical variables were made using Fisher's exact test. Mann-Whitney *U* test was used for comparing groups of continuous data sets. Spearman rank order correlation was used to assess correlations between continuous data.

Table 1. Clinical details of the tumor cases in this study

| Case no. | Sex-age (y) | Primary tumor | | Met/Rec Loc. inv. | Metastasis time (mo) | Follow-up | |
|-------------------------|-------------|---------------|------------------|-------------------|----------------------|-----------|--------|
| | | Size (cm) | Type | | | Time (y) | Status |
| <i>Malignant tumors</i> | | | | | | | |
| 1 | M-42 | 4.8 | Paraganglioma | Met, Rec | 41 | 13 | DOD |
| 2 | Met | | Met from no. 1 | | | | |
| 3 | F-41 | 7.2 | Paraganglioma | Met, Rec | 36 | 13 | DOD |
| 4 | Met | | Met from no. 3 | | | | |
| 5 | M-26 | 3.5 | Paraganglioma | Met, | At diagnosis | 2 | DOD |
| 6 | M-25 | 3.8 | Paraganglioma | Loc. inv., Rec | — | 16 | NED |
| 7 | M-40 | 10.5 | Pheochromocytoma | Met | At diagnosis | 0 | DOD |
| 8 | M-25 | 3.0 | Paraganglioma | Met | At diagnosis | 0 | DOR |
| 9 | M-13 | 5.0 | Pheochromocytoma | Loc. inv. | — | 13 | NED |
| 10 | F-77 | 10.0 | Pheochromocytoma | Loc. inv. | — | 7 | DOR |
| 11 | F-44 | 5.8 | Pheochromocytoma | Met, Rec | at Diagnosis | 11 | NED |
| 12 | F-64 | 4.2 | Pheochromocytoma | Loc. inv. | — | 9 | NED |
| 13 | F-69 | 3.5 | Pheochromocytoma | Loc. inv. | — | 9 | NED |
| <i>Benign tumors</i> | | | | | | | |
| 14 | M-14 | 4.0 | Paraganglioma | — | — | 20 | NED |
| 15 | F-57 | 6.5 | Paraganglioma | — | — | 15 | NED |
| 16 | M-47 | 2.5 | Paraganglioma | — | — | 9 | NED |
| 17 | F-38 | 8.5 | Pheochromocytoma | — | — | 22 | NED |
| 18 | F-58 | 13.0 | Pheochromocytoma | — | — | 21 | NED |
| 19 | F-22 | 5.8 | Pheochromocytoma | — | — | 21 | NED |
| 20 | F-46 | 9.5 | Pheochromocytoma | — | — | 21 | NED |
| 21 | F-69 | 3.5 | Pheochromocytoma | — | — | 9 | DOR |
| 22 | F-44 | 1.5 | Pheochromocytoma | — | — | 20 | NED |
| 23 | F-53 | 3.0 | Pheochromocytoma | — | — | 20 | NED |
| 24 | F-62 | 1.0 | Pheochromocytoma | — | — | 20 | DOR |
| 25 | M-56 | 1.5 | Pheochromocytoma | — | — | 20 | NED |
| 26 | M-58 | 1.6 | Pheochromocytoma | — | — | 19 | DOR |
| 27 | F-75 | 2.8 | Pheochromocytoma | — | — | 19 | DOR |
| 28 | M-44 | 3.2 | Pheochromocytoma | — | — | 18 | DOR |
| 29 | M-48 | 14.0 | Pheochromocytoma | — | — | 18 | NED |
| 30 | F-45 | 2.8 | Pheochromocytoma | — | — | 18 | NED |
| 31 | F-51 | 5.5 | Pheochromocytoma | — | — | 17 | NED |
| 32 | F-37 | 4.8 | Pheochromocytoma | — | — | 17 | NED |
| 33 | F-30 | 4.8 | Pheochromocytoma | — | — | 17 | NED |
| 34 | M-57 | 6.0 | Pheochromocytoma | — | — | 17 | NED |
| 35 | F-68 | 3.2 | Pheochromocytoma | — | — | 16 | DOR |
| 36 | M-31 | 3.5 | Pheochromocytoma | — | — | 14 | NED |
| 37 | F-74 | 4.0 | Pheochromocytoma | — | — | 13 | NED |
| 38 | F-64 | 5.5 | Pheochromocytoma | — | — | 12 | NED |
| 39 | F-51 | 3.5 | Pheochromocytoma | — | — | 12 | DOR |
| 40 | F-46 | 4.2 | Pheochromocytoma | — | — | 11 | NED |
| 41 | M-23 | 4.2 | Paraganglioma | — | — | 11 | NED |
| 42 | F-74 | 5.8 | Pheochromocytoma | — | — | 11 | NED |
| 43 | M-72 | 3.5 | Paraganglioma | — | — | 10 | NED |
| 44 | M-40 | 5.2 | Pheochromocytoma | — | — | 10 | NED |
| 45 | F-70 | 6.2 | Pheochromocytoma | — | — | 6 | DOR |
| 46 | F-52 | 3.8 | Pheochromocytoma | — | — | 10 | NED |
| 47 | M-54 | 2.5 | Pheochromocytoma | — | — | 10 | NED |
| 48 | M-59 | 5.0 | Pheochromocytoma | — | — | 9 | NED |
| 49 | F-61 | 5.5 | Pheochromocytoma | — | — | 9 | NED |
| 50 | F-50 | 1.8 | Pheochromocytoma | — | — | 8 | NED |
| 51 | F-73 | 5.0 | Pheochromocytoma | — | — | 7 | NED |
| 52 | M-52 | 7.0 | Pheochromocytoma | — | — | 6 | NED |
| 53 | M-76 | 4.2 | Pheochromocytoma | — | — | 6 | NED |
| 54 | M-66 | 3.2 | Pheochromocytoma | — | — | 6 | NED |
| 55 | M-27 | n/a | Pheochromocytoma | — | — | 3 | NED |

Abbreviations: NED, no evidence of disease; DOR, dead of other reasons; DOD, dead of disease; Met, metastasis; Rec, local recurrence; Loc. inv., local invasion.

Results

Quantification of CpG methylation in individual gene promoters. Fifty-three primary pheochromocytomas and paragangliomas and two corresponding paraganglioma metastases were

assessed for promoter methylation in *RARB*, *DCR2*, *CDH1*, *APC*, *TP73*, *DAPK1*, and *PTEN* (Supplementary Table SA). Normal adrenomedullary tissue samples analyzed in parallel

Table 2. Results from methylation quantifications in tumors

| Case no. | Tumor type | Met | Methylation by | | BRAF sequence | Z score | Gene-specific methylation | |
|-------------------------|------------|-----|----------------|-------|---------------|---------|---|------|
| | | | LINE-1 (%) | LUMA | | | Genes with methylation > CIMP cut-off | CIMP |
| <i>Malignant tumors</i> | | | | | | | | |
| 1 | Paraga | Met | 63.4 | 0.332 | wt | 2.48 | <i>DCR2, RASSF1A, p16, RARB, CDH1</i> | CIMP |
| 2 ^{Met} | Paraga | Met | 64.5 | 0.324 | wt | 3.20 | <i>DCR2, RASSF1A, p16, RARB, CDH1</i> | CIMP |
| 3* | Paraga | Met | 63.6 | 0.799 | wt | 2.42 | <i>DCR2, RASSF1A, p16, NORE1A</i> | CIMP |
| 4 ^{Met*} | Paraga | Met | 57.0 | 0.438 | wt | 2.23 | <i>DCR2, RASSF1A, p16, NORE1A, RARB</i> | CIMP |
| 5* | Paraga | Met | 62.7 | n/a | wt | 1.37 | <i>DCR2, RASSF1A, p16</i> | CIMP |
| 6* | Paraga | — | 63.0 | 0.399 | wt | 0.30 | <i>DCR2, RASSF1A, p16</i> | CIMP |
| 7 | Pheo | Met | 61.5 | 0.412 | wt | 0.12 | <i>RASSF1A</i> | — |
| 8 | Paraga | Met | 64.2 | 0.434 | wt | -0.45 | — | — |
| 9 | Pheo | — | 67.2 | 0.362 | wt | -0.46 | — | — |
| 10 | Pheo | — | 63.1 | 0.381 | wt | -0.38 | — | — |
| 11 | Pheo | Met | 54.5 | 0.443 | wt | -0.36 | — | — |
| 12 | Pheo | — | 63.4 | 0.381 | wt | -0.20 | — | — |
| 13 | Pheo | — | 51.4 | 0.494 | wt | -0.35 | <i>RASSF1A</i> | — |
| <i>Benign tumors</i> | | | | | | | | |
| 14* | Paraga | — | 65.9 | 0.244 | wt | 1.74 | <i>DCR2, RASSF1A, p16, RARB</i> | CIMP |
| 15 | Paraga | — | 67.4 | 0.348 | wt | 0.35 | <i>CDH1</i> | — |
| 16 | Paraga | — | 63.6 | 0.440 | wt | -0.14 | <i>RASSF1A</i> | — |
| 17 | Pheo | — | 43.6 | n/a | wt | -0.45 | — | — |
| 18 | Pheo | — | 64.9 | n/a | wt | 0.06 | — | — |
| 19 | Pheo | — | 59.6 | n/a | wt | -0.37 | — | — |
| 20 | Pheo | — | 65.1 | n/a | wt | -0.38 | — | — |
| 21 | Pheo | — | 69.5 | n/a | wt | -0.08 | — | — |
| 22 | Pheo | — | 57.8 | 0.395 | wt | -0.28 | — | — |
| 23 | Pheo | — | 65.2 | n/a | wt | -0.19 | — | — |
| 24 | Pheo | — | 68.7 | n/a | wt | -0.26 | — | — |
| 25 | Pheo | — | 65.1 | 0.339 | wt | -0.12 | — | — |
| 26 | Pheo | — | 61.9 | 0.500 | wt | -0.32 | — | — |
| 27 | Pheo | — | 67.7 | n/a | wt | -0.24 | — | — |
| 28 | Pheo | — | 63.7 | n/a | wt | -0.36 | — | — |
| 29 | Pheo | — | 63.1 | n/a | wt | -0.40 | — | — |
| 30 | Pheo | — | 62.5 | n/a | wt | -0.37 | — | — |
| 31 | Pheo | — | 54.8 | n/a | wt | -0.01 | — | — |
| 32 | Pheo | — | 56.6 | n/a | wt | -0.11 | — | — |
| 33 | Pheo | — | 62.3 | n/a | wt | -0.58 | — | — |
| 34 | Pheo | — | 68.0 | 0.368 | wt | -0.21 | — | — |
| 35 | Pheo | — | 58.3 | 0.433 | wt | -0.52 | — | — |
| 36 | Pheo | — | 68.7 | n/a | wt | -0.37 | — | — |
| 37 | Pheo | — | 62.5 | 0.398 | wt | -0.09 | — | — |
| 38 | Pheo | — | n/a | 0.480 | wt | -0.58 | — | — |
| 39 | Pheo | — | 62.7 | 0.382 | wt | -0.09 | <i>RASSF1A</i> | — |
| 40 | Pheo | — | 64.4 | 0.382 | wt | -0.29 | — | — |
| 41 | Paraga | — | 61.9 | 0.566 | wt | -0.25 | — | — |
| 42 | Pheo | — | 66.0 | 0.344 | wt | -0.09 | <i>CDH1</i> | — |
| 43 | Paraga | — | 64.3 | 0.377 | wt | -0.27 | — | — |
| 44 | Pheo | — | 66.5 | n/a | wt | -0.43 | — | — |
| 45 | Pheo | — | 52.6 | n/a | wt | -0.41 | — | — |
| 46 | Pheo | — | 56.6 | n/a | wt | -0.42 | — | — |
| 47 | Pheo | — | 55.1 | 0.426 | wt | -0.34 | — | — |
| 48 | Pheo | — | 63.5 | 0.378 | wt | -0.12 | <i>RASSF1A</i> | — |
| 49 | Pheo | — | 57.1 | 0.475 | wt | -0.39 | — | — |
| 50 | Pheo | — | 55.3 | 0.508 | wt | -0.37 | — | — |
| 51 | Pheo | — | 69.2 | n/a | wt | 0.93 | <i>RASSF1A, APC</i> | — |
| 52 | Pheo | — | 60.8 | 0.791 | wt | -0.45 | — | — |
| 53 | Pheo | — | 64.9 | n/a | wt | -0.38 | — | — |
| 54 | Pheo | — | 55.9 | 0.885 | wt | -0.33 | — | — |
| 55 | Pheo | — | 67.5 | n/a | wt | -0.10 | <i>RASSF1A</i> | — |

NOTE: Data for *RASSF1A*, *NORE1A*, and *p16* are from ref. 26 and Kiss et al.³

Abbreviations: Paraga, abdominal paraganglioma; Pheo, pheochromocytoma; n/a, not analyzed or not available; —, not detected; wt, wild type.

*Indicates tumors with *SDHB* mutation.

Table 3. Summary of gene-specific promoter methylation analyses

| Gene studied | Assayed in | Annealing at PCR (°C) | Sequence entry | No. CpGs assayed | Methylation in normal (%) | Methylation in tumors | | CIMP cutoff | | CIMP phenotype | |
|-----------------|--------------------------|-----------------------|----------------|------------------|---------------------------|-----------------------|-----------|-------------|-----|----------------|-----------|
| | | | | | | Mean (%) | (Range) | Above (%) | No. | Present | Range (%) |
| <i>APC</i> | This study | 55 | U02509 | 10 | <3.5 | 0.9 | (0-19) | >10 | 1 | No | — |
| <i>CDH1</i> | This study | 53 | L34545 | 9 | <4.6 | 4.9 | (1.5-25) | >10 | 4 | CIMP | (10-25) |
| <i>DAPK1</i> | This study | 55 | AL161787 | 13 | <1.2 | 0.4 | (0-2.2) | >10 | 0 | No | — |
| <i>PTEN</i> | This study | 53 | AF143312 | 10 | <3.2 | 0.6 | (0.1-1.9) | >10 | 0 | No | — |
| <i>RARB</i> | This study | 50 | AC098477.2 | 10 | <7.3 | 3.2 | (0.3-30) | >10 | 4 | CIMP | (14-30) |
| <i>TP73</i> | This study | 55 | AL136528 | 19 | <2.0 | 0.6 | (0.0-3.0) | >10 | 0 | No | — |
| <i>p14ARF</i> | Kiss et al. ³ | 53 | — | 13 | <3.4 | 1.4 | (0.4-4.8) | >10 | 0 | No | — |
| <i>p16INK4A</i> | Kiss et al. ³ | 55 | — | 4 | <1.4 | 6.8 | (0.0-65) | >10 | 7 | CIMP | (22-65) |
| <i>NORE1A</i> | Geli et al. (26) | 53 | — | 13 | <2.8 | 1.4 | (0.0-20) | >10 | 2 | CIMP | (12-20) |
| <i>RASSF1A</i> | Geli et al. (26) | 55 | — | 5 | <6.5 | 11 | (0.3-57) | >10 | 14 | CIMP | (15-57) |

NOTE: Methylation levels represent mean values for the CpGs assessed.

revealed low levels of methylation (Table 3). Methylation above 10% was therefore chosen as a cutoff value for hypermethylated status of all genes, with the exception of *DCR2* for which 30% was used as a cutoff. In the tumors, *TP73*, *DAPK1*, and *PTEN* only revealed low levels of methylation, which were considered to constitute background levels with questionable biological significance. By contrast, the promoters of *RARB*, *DCR2*, *CDH1*, and *APC* were hypermethylated in subsets of the tumors, as exemplified for case 1 in Fig. 1. These results were subsequently combined with previously reported analyses of *p16^{INK4A}*, *p14^{ARF}*, *RASSF1A*, and *NORE1A* to define tumors with a hypermethylated phenotype. Taken together, 14 primary tumors (six malignant, eight benign) and the two metastases showed significant methylation in one or more promoters of the genes *DCR2*, *RASSF1A*, *NORE1A*, *p16^{INK4A}*, *RARB*, *CDH1*, and *APC* (Tables 2 and 3).

CIMP phenotype in association with malignancy. Five of 53 primary tumors (9.4%) showed hypermethylation in at least three promoters, fulfilling the previously defined criteria for a CIMP phenotype (Table 2). Four primary tumors with CIMP were malignant paragangliomas, of which three developed distant metastases (Tables 1 and 2) and one tumor was a benign paraganglioma. CIMP was significantly associated with malignant behavior (Fisher's exact test, $P = 0.005$). In the cases of matched primary tumors and metastases CIMP was observed both in the primary tumors and the corresponding metastases with a highly similar pattern of hypermethylated promoters. Significantly younger age of presentation was detected in CIMP cases compared with those without CIMP ($P < 0.007$; mean, 29 versus 52 years; Fig. 2A), whereas, overall, no difference in age at presentation was shown between malignant and benign cases. The only benign case classified as CIMP presented with paraganglioma at an unusually young age of 14 years.

Association between high Z score and malignancy. Z scores were calculated based on the seven genes for which hypermethylation was detected in the tumor panel i.e., *DCR2*, *RASSF1A*, *NORE1A*, *p16^{INK4A}*, *RARB*, *CDH1*, *APC* (Table 2). Mean Z scores for primary tumors ranged from -0.58 to 2.48 with an average for the whole set of 0.06. Significantly higher Z score was observed among primary tumors with CIMP compared

with those without CIMP ($P < 0.001$; means, 1.66 versus -0.24; Fig. 2B). A total of 10 primary tumors (six malignant, four benign) and the two metastases showed a mean Z score of >0 , which was significantly associated with malignant behavior ($P = 0.02$, Fisher's exact test). Samples with the 10 highest mean Z score included all tumors previously classified as CIMP. For the matched primary tumors and metastases, mean Z scores were 2.5 versus 3.2 (cases 1 and 2) and 2.4 versus 2.2 (cases 3 and 4). Taking into account the entire primary tumor population, no statistically significant difference was shown compared with normal controls with regard to mean Z score. The observation that only paragangliomas showed CIMP was reflected by higher Z score in paragangliomas compared with pheochromocytomas ($P < 0.005$). No other association was observed between Z score and clinical characteristics.

Global CpG methylation of LINE-1 promoter elements. Overall lower levels of *LINE-1* methylation were observed in tumors compared with normal adrenal samples (Supplementary Table SB; Table 2; Fig. 2C; Mann-Whitney U test, $P < 0.02$, 0.632 versus 0.674). With regard to the individual gene promoters assessed, a weak correlation was observed between methylation density of *LINE-1* and *p16^{INK4A}*, as well as *DCR2* (Spearman rank order correlations, 0.29 and 0.36, respectively; $P < 0.05$), which was also reflected by a similar relationship between *LINE-1* mean Z scores (Spearman rank order correlation, 0.29; $P < 0.05$). However, no significant difference was observed between CIMP and non-CIMP tumors with regard to global methylation by *LINE-1*. Neither was there an association between *LINE-1* methylation and other clinical characteristics. Taken together, these results suggest that a modest degree of global CpG hypomethylation is frequent in these tumors, but is largely independent of the CIMP phenotype and hypermethylation of individual tumor suppressor genes (Fig. 3).

Global methylation assessed by LUMA. No significant differences were observed in global methylation determined by LUMA between tumors and normal control samples. Although the tumors exhibited large variations in *HpaII/MspI* ratios, this was not associated with the clinical tumor features or with CIMP (Fig. 3). A negative association was observed between

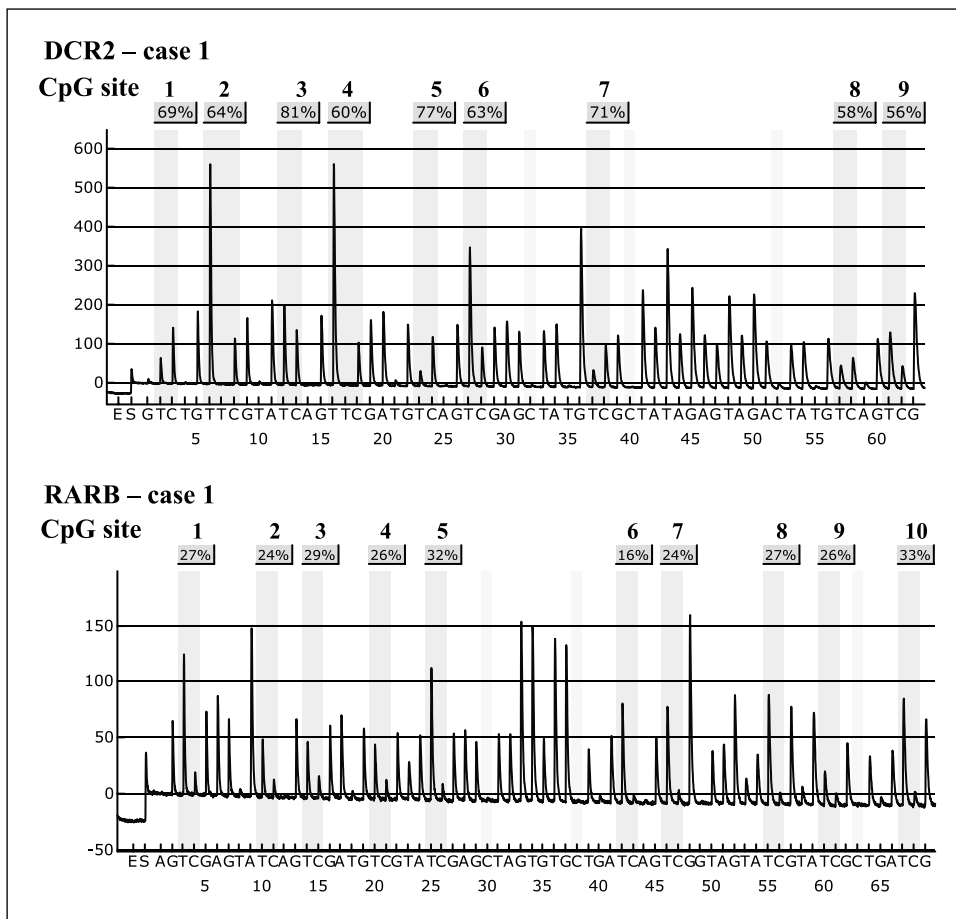


Fig. 1. Examples of pyrograms for quantification of CpG methylation in case no. 1 with a CIMP phenotype. The pyrograms for *DCR2* and *RARB* exhibit hypermethylation of mean densities of 66.5% and 26.5%, respectively.

mean Z scores and *HpaII/MspI* ratios (Spearman rank order correlation, -0.48 ; $P < 0.05$), which was further accentuated when only benign samples were considered (Spearman rank order correlation, -0.74 ; $P < 0.05$). *HpaII/MspI* ratios and *LINE-1* methylation were negatively correlated (Spearman rank order correlation, -0.69 $P < 0.05$).

Discussion

In the present study, we quantitatively assessed promoter methylation density of tumor suppressor genes with a broad spectrum of cellular functions and frequent hypermethylation in cancer. Fourteen primary tumors (six malignant, eight

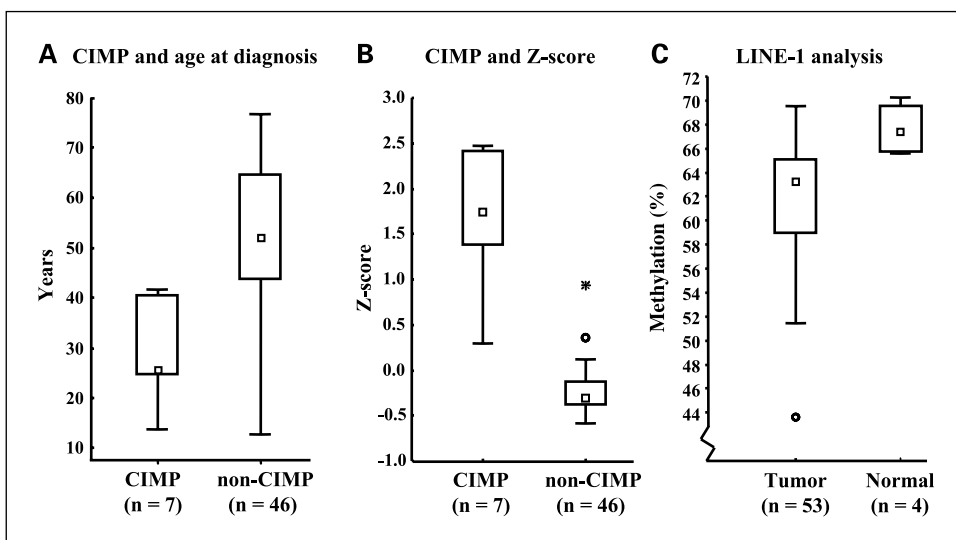


Fig. 2. Box plots showing lower age at diagnosis for patients with CIMP (A), association between CIMP phenotype and high Z scores (B), and global CpG hypomethylation of *LINE-1* promoter sequences in tumors compared with normal adrenal medullary samples (C).

benign) and the two distant metastases were significantly methylated at one or more of the following promoters: *RASSF1A*, *NORE1A*, *p16^{INK4A}*, *RARB*, *DCR2*, *CDH1*, and *APC*. Importantly, simultaneous methylation of three or more promoters, here defined as CIMP, was confined to a subset of five primary paragangliomas, four of which were malignant (Tables 1 and 2). Notably, three of these paraganglioma cases developed distant metastases, and one showed recurrence on the contralateral side after primary surgery. Thus, CIMP was strongly associated with malignant disease ($P = 0.005$). This correlation was also well reflected by the overrepresentation of malignant cases among tumors with a mean Z score of >0 ($P = 0.02$). Interestingly, none of the pheochromocytoma cases were classified as CIMP, which further illustrates the molecular differences between these, in many other aspects highly similar to chromaffin tumor entities (35).

Further studies are required to unravel whether CIMP is a causative factor or a consequence of the malignification process. Association of CIMP with adverse clinical behavior, such as high stage and development of distant metastases, has been described in some but not all tumor types examined (4, 8, 11, 14, 17, 18) and may reflect a selection for CIMP phenotypes during malignant transformation (29). As cytosine methylation in CpG dense promoters constitutes a major epigenetic mechanism of transcriptional silencing (1), it is plausible that one of the ways by which CIMP may contribute to the development of neoplastic phenotype is realized through simultaneous transcriptional suppression of multiple genes governing proliferation, cell death, and invasion. Given the heterogeneity in tumors, hypermethylation may only be present in a subset of cells leading to transcriptional suppression in only a minority of selected cells (indeed 100% methylation density is rarely seen in biological specimens). Several of the genes analyzed in here show suppressed expression in various neoplasias, among them in pheochromocytomas and abdominal paragangliomas (26). As promoter hypermethylation is only one of the possible mechanisms resulting in mRNA suppression, even tumors without high levels of methylation may exhibit expressional suppression by other means.

The two distant metastases and primary tumor counterparts exhibited consistent patterns of hypermethylation, suggesting that CIMP have preceded metastasis development. A potential implication of this is that the presence of CIMP in the primary lesion may indicate an increased likelihood of metastasis development at a later time point, a notion that requires further substantiation on extended sets of matched primary and metastatic lesions.

Given that *de novo* DNA methylation and its spreading is a replication-dependent phenomenon (42), promoter methylation might also be a reflection of the past replication history of the cells. This may certainly be true for many genes; however, if CIMP was merely a mirror of past cell divisions, one would anticipate that CIMP tumors have a larger size and/or present in older individuals-which could not be supported by our data. On the other hand, our previously reported high proliferation index in a subset of these tumors (43) largely overlap with the CIMP phenotype identified in this study.

At present, little is known about the triggering factors facilitating the emergence of CIMP in cancer. Concordant methylation in multiple genes may suggest a primary aberration

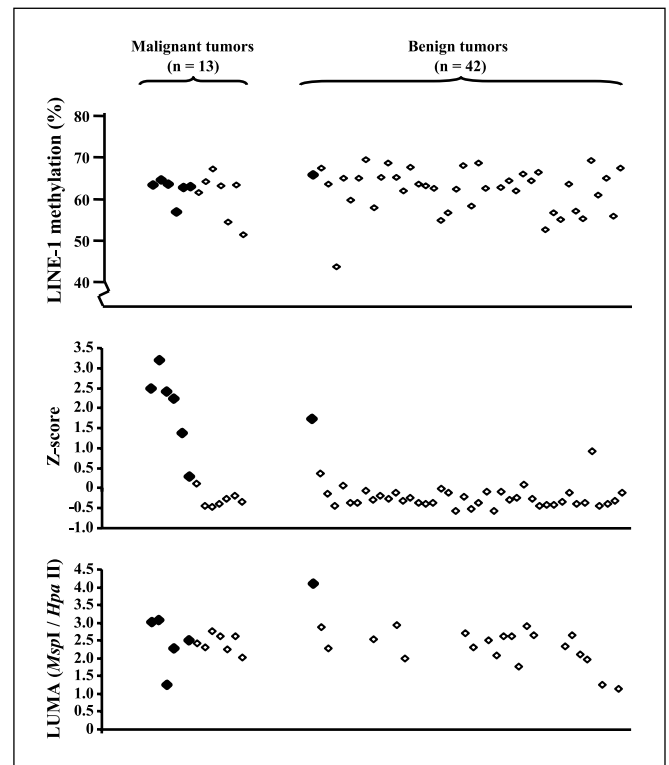


Fig. 3. Comparison of global methylation levels in *LINE-1* elements and determined by LUMA and Z scores for gene-specific methylation measurements in tumor samples. Each sample is represented by a filled or open symbol according to presence or absence of CIMP phenotype. Tumors are organized in increasing case number order. LUMA results are graphically presented as *MspI/HpaII* ratios, whereby higher values signifies comparatively higher degrees of methylation, thus simplifying comparisons to other assays shown.

tion of pathways directly involved in DNA methylation. Such event could possibly be the deregulation of DNA methyl transferases or histone deacetylases. Alternatively, activation of other oncogenic cascades or inactivation of tumor suppressor pathways may indirectly affect DNA methylation. Consistent with this concept are reports showing substantial association of CIMP with previously well-described molecular alterations, such as *BRAF* V600E mutations in colorectal cancer or *MYCN* amplification in neuroblastoma (8, 12, 14). In our tumor panel, we did not detect any *BRAF* V600E mutation. However, screening for mutations in other genes associated with this tumor type, such as *RET*, *VHL*, *SDHD*, and *SDHB* (22), is highly relevant. The association of *SDHB* mutations with an increased frequency of malignant behavior in pheochromocytomas and paragangliomas (44) makes this gene a particularly attractive candidate for mutation screening. Mutation analysis of the *SDHB* gene was done in tumors from cases classified as malignant, as paragangliomas and tumors were diagnosed before 30 years of age.³ This revealed mutations in tumors 3, 4, 5, 6, and 14 (Table 2).

All these cases were paragangliomas exhibiting CIMP phenotype. A possible link between the *SDHB* gene product and the DNA methylation machinery is yet to be unraveled.

Studies using global gene expression profiling and array CGH may greatly aid to further dissect the molecular features that discern CIMP-positive paragangliomas from their non-CIMP counterparts. This in turn may enhance our understanding of

the triggering causes of CIMP and the mechanisms by which it may contribute to aggressive behavior and early presentation in paragangliomas.

Reports on increased cancer prevalence among first-degree relatives of patients with CIMP colonic lesions (15, 16) raise a prospect for the presence of constitutional mutations underlying CIMP. Thus, it is speculated that several, yet unidentified, familial cancer syndromes may harbor such etiology (29). In support of this notion younger age at presentation was observed in CIMP tumors compared with non-CIMP cases (29 versus 52 years, $P < 0.007$; Fig. 2A), which is a well-described feature of the familial form of the disease (22). Interestingly the only benign tumor showing CIMP presented at an unusually young age of 14 years. Therefore, detailed evaluation of cancer incidence in the pedigrees of CIMP paraganglioma cases should be addressed on larger series together with screening for mutations in candidate genes.

The lack of consensus definition for CIMP reflects our fragmentary understanding of the multifaceted features of dysregulation in the methylation machinery observed in cancer. To further explore the prevalence and potential clinical usefulness of this molecular phenotype, it is imperative to define standardized ways to measure and uniformly report methylation changes in multiple promoters. There is great variation with regard to the specific set of promoters reported to be hypermethylated in other tumor types with CIMP, which probably reflects tissue-specific variation of DNA methylation (29). Quantitative detection of methylation involving an expanded set of genes in a large tumor series would aid to further outline optimum criteria for the definition of CIMP in paragangliomas. It is crucial if CIMP is to be evaluated as a potential prognostic marker suitable for improved risk stratification of paraganglioma patients (i.e., predicting the likelihood of malignant behavior). Given the scarcity of this tumor type, this could be best achieved in the setting of a collaborative group study on a more comprehensive scale.

The prognosis of patients with malignant paraganglioma is dismal (35). Therefore, new approaches in the treatment of affected patients are urgently needed. Here, we have shown an

association of CIMP status with malignant behavior and young age at presentation in paragangliomas. As the hallmark of CIMP is the hypermethylation of multiple tumor suppressor promoters, epigenetically acting drugs, such as inhibitors of DNA methylation, which are already approved for the treatment of some neoplasias, may be highly relevant candidates for testing as adjuvant treatments in CIMP malignant paragangliomas.

Besides gene-specific methylation, global CpG methylation has also been characterized in the same tumor set. A recent study by Choi et al. showed global hypomethylation in pancreatic and intestinal neuroendocrine tumors compared with normal tissue using *LINE-1* as a surrogate for genome-wide methylation (45). Here, we provide a similar observation in pheochromocytomas and paragangliomas. However, when applying a different methodology, which essentially assesses methylation density at CCGG restriction sites across the genome (LUMA), no such difference could be shown. This further underlines that interrogation of different sequences as surrogate markers for global methylation explore distinct components of genome-wide methylation, which may result in slightly divergent findings. Overall however, there was a correlation between global methylation values obtained by LUMA (expressed as *HpaII/MspI* ratios) and *LINE-1*. Furthermore, no association was revealed with promoter-specific methylation or other clinical characteristics, suggesting that alterations in global methylation levels are more of a general feature of chromaffin cancer development.

In conclusion, we here report the identification of CIMP in paragangliomas, showing strong correlation with malignant behavior and young age at presentation. Given the potential diagnostic and therapeutic implications that CIMP may entail for these young patients, further genetic and epigenetic studies using larger tumor sets are urgently warranted.

Acknowledgments

We thank Drs. Bertil Hamberger, Elisabeth Edström-Elder, and Anders Höög for their clinical expertise and Lisa Åhnfalk for the excellent support in tumor sample handling.

References

- Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. *Science* 2001;293:1068–70.
- Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer* 2004;4:143–53.
- Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999;96:8681–6.
- Brock MV, Gou M, Akiyama Y, et al. Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. *Clin Cancer Res* 2003;9:2912–9.
- Shen L, Ahuja N, Shen Y, et al. DNA methylation and environmental exposures in human hepatocellular carcinoma. *J Natl Cancer Inst* 2002;94:755–61.
- Toyota M, Ahuja N, Suzuki H, et al. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999;59:5438–42.
- Ueki T, Toyota M, Sohn T, et al. Hypermethylation of multiple genes in pancreatic adenocarcinoma. *Cancer Res* 2000;60:1835–9.
- Abe M, Westermann F, Nakagawara A, Takato T, Schwab M, Ushijima T. Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas. *Cancer Lett* 2007;247:253–8.
- Li Q, Jedlicka A, Ahuja N, et al. Concordant methylation of the ER and N33 genes in glioblastoma multiforme. *Oncogene* 1998;16:3197–202.
- Garcia-Manero G, Daniel J, Smith TL, et al. DNA methylation of multiple promoter-associated CpG islands in adult acute lymphocytic leukemia. *Clin Cancer Res* 2002;8:2217–24.
- Roman-Gomez J, Jimenez-Velasco A, Agirre X, et al. CpG island methylator phenotype redefines the prognostic effect of t(12;21) in childhood acute lymphoblastic leukemia. *Clin Cancer Res* 2006;12:4845–50.
- Kambara T, Simms LA, Whitehall VL, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 2004;53:1137–44.
- Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci U S A* 2000;97:710–5.
- Abe M, OHIRA M, Kaneda A, et al. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res* 2005;65:828–34.
- Frazier ML, Xi L, Zong J, et al. Association of the CpG island methylator phenotype with family history of cancer in patients with colorectal cancer. *Cancer Res* 2003;63:4805–8.
- Ricciardiello L, Goel A, Mantovani V, et al. Frequent loss of hMLH1 by promoter hypermethylation leads to microsatellite instability in adenomatous polyps of patients with a single first-degree member affected by colon cancer. *Cancer Res* 2003;63:787–92.
- Shaw RJ, Hall GL, Lowe D, et al. CpG island methylation phenotype (CIMP) in oral cancer: associated with a marked inflammatory response and less aggressive tumour biology. *Oral Oncol* 2007;43:878–86.
- Ward RL, Cheong K, Ku SL, Meagher A, O'Connor T, Hawkins NJ. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. *J Clin Oncol* 2003;21:3729–36.

19. Estecio MR, Gharibyan V, Shen L, et al. LINE-1 Hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. *PLoS ONE* 2007;2:e3399.
20. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983;301:89–92.
21. Elder EE, Elder G, Larsson C. Pheochromocytoma and functional paraganglioma syndrome: no longer the 10% tumor. *J Surg Oncol* 2005;89:193–201.
22. Neumann HP, Bausch B, McWhinney SR, et al. Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med* 2002;346:1459–66.
23. Astuti D, Agathangelou A, Honorio S, et al. RASSF1A promoter region CpG island hypermethylation in pheochromocytomas and neuroblastoma tumours. *Oncogene* 2001;20:7573–7.
24. Cascon A, Ruiz-Llorente S, Fraga MF, et al. Genetic and epigenetic profile of sporadic pheochromocytomas. *J Med Genet* 2004;41:e30.
25. Dammann R, Schagdarsurengin U, Seidel C, et al. Frequent promoter methylation of tumor-related genes in sporadic and men2-associated pheochromocytomas. *Exp Clin Endocrinol Diabetes* 2005;13:1–7.
26. Geli J, Kiss N, Lanner F, et al. The Ras effectors NORE1A and RASSF1A are frequently inactivated in pheochromocytoma and abdominal paraganglioma. *Endocr Relat Cancer* 2007;14:125–34.
27. Margetts CD, Astuti D, Gentle DC, et al. Epigenetic analysis of HIC1, CASP8, FLIP, TSP1, DCR1, DCR2, DR4, DR5, KvDMR1, H19 and preferential 11p15.5 maternal-allele loss in von Hippel-Lindau and sporadic pheochromocytomas. *Endocr Relat Cancer* 2005;12:161–72.
28. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996;93:9821–6.
29. Issa JP. CpG island methylator phenotype in cancer. *Nat Rev Cancer* 2004;4:988–93.
30. Dupont JM, Tost J, Jammes H, Gut IG. *De novo* quantitative bisulfite sequencing using the pyrosequencing technology. *Anal Biochem* 2004;333:119–27.
31. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, et al. Phase 1/2 study of the combination of 5-aza-2'-deoxycytidine with valproic acid in patients with leukemia. *Blood* 2006;108:3271–9.
32. Kang S, Kim J, Kim HB, et al. Methylation of p16INK4a is a non-rare event in cervical intraepithelial neoplasia. *Diagn Mol Pathol* 2006;15:74–82.
33. Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. *J Mol Diagn* 2007;9:305–14.
34. Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 2006;55:1000–6.
35. Edström Elder E, Hjelm Skog A, Höög A, Hamberger B. The management of benign and malignant pheochromocytoma and abdominal paraganglioma. *Eur J Surg Oncol* 2003;29:278–83.
36. Lack E. Tumors of the adrenal gland and extra-adrenal paraganglia. In: *Armed Forces Institute of Pathology, Washington (DC)*; 1997.
37. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 2004;32:e38.
38. Karimi M, Johansson S, Stach D, et al. LUMA (Luminometric Methylation Assay) - a high throughput method to the analysis of genomic DNA methylation. *Exp Cell Res* 2006;312:1989–95.
39. Lee JJ, Foukakis T, Hashemi J, et al. Molecular cytogenetic profiles of novel and established human anaplastic thyroid carcinoma models. *Thyroid* 2007;17:289–301.
40. Nikiforova MN, Kimura ET, Gandhi M, et al. BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. *J Clin Endocrinol Metab* 2003;88:5399–404.
41. Konishi K, Shen L, Wang S, Meltzer SJ, Harpaz N, Issa JP. Rare CpG island methylator phenotype in ulcerative colitis-associated neoplasias. *Gastroenterology* 2007;132:1254–60.
42. Velicescu M, Weisenberger DJ, Gonzales FA, Tsai YC, Nguyen CT, Jones PA. Cell division is required for *de novo* methylation of CpG islands in bladder cancer cells. *Cancer Res* 2002;62:2378–84.
43. Elder EE, Xu D, Höög A, et al. KI-67 and hTERT expression can aid in the distinction between malignant and benign pheochromocytoma and paraganglioma. *Mod Pathol* 2003;16:246–55.
44. Neumann HP, Pawlu C, Peczkowska M, et al. Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations. *JAMA* 2004;292:943–51.
45. Choi IS, Estecio MR, Nagano Y, et al. Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod Pathol* 2007;20:802–10.

Clinical Cancer Research

Global and Regional CpG Methylation in Pheochromocytomas and Abdominal Paragangliomas: Association to Malignant Behavior

Janos Geli, Nimrod Kiss, Mohsen Karimi, et al.

Clin Cancer Res 2008;14:2551-2559.

| | |
|-------------------------------|---|
| Updated version | Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/14/9/2551 |
| Supplementary Material | Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2008/07/11/14.9.2551.DC1 |

| | |
|------------------------|---|
| Cited articles | This article cites 43 articles, 19 of which you can access for free at: http://clincancerres.aacrjournals.org/content/14/9/2551.full#ref-list-1 |
| Citing articles | This article has been cited by 5 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/14/9/2551.full#related-urls |

| | |
|-----------------------------------|--|
| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org . |
| Permissions | To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/14/9/2551 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site. |