

The *VHL* gene is epigenetically inactivated in pheochromocytomas and abdominal paragangliomas

Adam Andreasson^{1*}, Nimrod B Kiss¹, Stefano Caramuta¹, Luqman Sulaiman¹, Fredrika Svahn¹, Martin Bäckdahl^{2,3}, Anders Höög^{1,4}, C Christofer Juhlin^{1,4}, and Catharina Larsson¹

¹Department of Oncology-Pathology; Karolinska Institutet; Karolinska University Hospital; Stockholm, Sweden; ²Department of Molecular Medicine and Surgery; Karolinska Institutet; Stockholm, Sweden; ³Department of Breast and Endocrine Surgery; Karolinska University Hospital; Stockholm, Sweden;

⁴Department of Clinical Pathology and Cytology; Karolinska University Hospital; Stockholm, Sweden

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Abbreviations: AFIP, armed forces institute of pathology; bp, base pair; CIMP, CpG island methylator phenotype; *EGLN1* (*PDH2*), egl nine homolog 1 gene; *EPAS* (*HIF2A*), endothelial PAS domain 1 gene; *KIF1B* β , kinesin family member 1B beta gene; *MAX*, myc associated factor X gene; MEN 2A, multiple endocrine neoplasia type 2A; MetI, methylation index; NF1, neurofibromatosis type 1 syndrome; *NFI*, neurofibromin 1 gene; PCC, pheochromocytoma; PGL, abdominal paraganglioma; *RET*, rearranged during transfection proto-oncogene; *SDHA*, succinate dehydrogenase complex, subunit A gene; *SDHAF2* (*SDH5*), succinate dehydrogenase complex assembly factor 2 gene; *SDHB* succinate dehydrogenase complex, subunit B gene; *SDHC* succinate dehydrogenase complex, subunit C gene; *SDHD* succinate dehydrogenase complex, subunit D gene; *TMEM127*, transmembrane protein 127 gene; *VHL*, von Hippel-Lindau syndrome; *VHL*, von Hippel-Lindau tumor suppressor gene; WHO, world health organization

Pheochromocytoma (PCC) and abdominal paraganglioma (PGL) are neuroendocrine tumors that present with clinical symptoms related to increased catecholamine levels. About a third of the cases are associated with constitutional mutations in pre-disposing genes, of which some may also be somatically mutated in sporadic cases. However, little is known about inactivating epigenetic events through promoter methylation in these very genes. Using bisulphite pyrosequencing we assessed the methylation density of 11 PCC/PGL disease genes in 96 tumors (83 PCCs and 13 PGLs) and 34 normal adrenal references. Gene expression levels were determined by quantitative RT-PCR. Both tumors and normal adrenal samples exhibited low methylation index (MetI) in the *EGLN1* (*PDH2*), *MAX*, *MEN1*, *NF1*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2* (*SDH5*), and *TMEM127* promoters, not exceeding 10% in any of the samples investigated. Aberrant *RET* promoter methylation was observed in two cases only. For the *VHL* gene we found increased MetI in tumors as compared with normal adrenals (57% vs. 27%; $P < 0.001$), in malignant vs. benign tumors (63% vs. 55%; $P < 0.05$), and in PGL vs. PCC (66% vs. 55%; $P < 0.0005$). Decreased expression of the *VHL* gene was observed in all tumors compared with normal adrenals ($P < 0.001$). *VHL* MetI and gene expressions were inversely correlated ($R = -0.359$, $P < 0.0001$). Our results show that the *VHL* gene promoter has increased methylation compared with normal adrenals (MetI > 50%) in approximately 75% of PCCs and PGLs investigated, highlighting the role of *VHL* in the development of these tumors.

Introduction

Pheochromocytoma (PCC) is a neuroendocrine tumor located in the adrenal medulla. Abdominal paraganglioma (PGL) is a highly related tumor occurring outside the adrenal medulla, most commonly within the sympathetic paravertebral ganglia.

The clinical profile of PCC and PGL is often characterized by increased catecholamine secretion and associated symptoms—most pronounced in tumors with epinephrine overexpression.¹

The majority of patients present with common endocrine-related manifestations such as headache, palpitations, tachycardia and excessive perspiration,² but asymptomatic tumors are also common and many are found only at autopsy.³

The last few decades have witnessed major discoveries relating to the genetic background of these tumors. Approximately one third of PCC and PGL patients carry a constitutional mutation in a predisposing gene including *EGLN1* (*PDH2*), *EPAS1* (*HIF2A*), *KIF1B* β , *MAX*, *NF1*, *RET*, *SDHA*, *SDHAF2* (*SDH5*), *SDHB*, *SDHC*, *SDHD*, *TMEM127*, and *VHL*.⁴⁻⁹ Somatic

*Correspondence to: Adam Andreasson; Email: Adam.Andreasson@ki.se
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Table 1. Results of methylation analyses in the tumors and normal adrenals

Gene symbol	Gene name	Tumors			Adrenals			Tumors vs. Adrenals <i>P</i> value
		n	MetI mean %	MetI range %	n	MetI mean %	MetI range %	
<i>EGLN1</i>	Egl nine homolog 1	95	1.6	0–5.6	12	1.2	0–2.4	n.s.
<i>MAX</i>	Myc associated factor X	96	1.0	0–4.7	6	0.9	0–3.2	n.s.
<i>MEN1</i>	Multiple endocrine neoplasia type 1	95	1.6	0–5.7	11	1.6	0.9–4.6	n.s.
<i>NF1</i>	Neurofibromin 1	96	1.0	0–3.8	4	0.6	0–1.5	n.s.
<i>RET</i>	Rearranged during transfection proto-oncogene	96	2.8	0–28.8	12	2.9	1.0–3.8	n.s.
<i>SDHAF2</i>	Succinate dehydrogenase complex assembly factor 2	96	0.8	0–4.0	12	0.8	0–2.5	n.s.
<i>SDHB</i>	Succinate dehydrogenase complex, subunit B	93	1.1	0–1.7	15	1.5	0.6–2.0	n.s.
<i>SDHC</i>	Succinate dehydrogenase complex, subunit C	95	0.3	0–1.5	18	0.6	0–2.3	n.s.
<i>SDHD</i>	Succinate dehydrogenase complex, subunit D	96	1.5	0–3.5	15	0.7	0–1.5	n.s.
<i>TMEM127</i>	Transmembrane protein 127	96	1.5	0–9.0	11	2.3	0.9–5.0	n.s.
<i>VHL</i>	Von Hippel-Lindau tumor suppressor	96	56.5	19.1–88.6	34	27.0	14.4–43.8	<i>P</i> < 0.0001

n.s., not statistically significant; MetI, methylation index

mutations in some of these genes also occur, most commonly involving *NF1*.^{10,11}

Studies of DNA methylation have revealed CpG hypermethylator phenotypes in PGLs carrying a *SDHB*,^{12,13} or *SDHx* mutation as was also demonstrated in a mouse model.¹⁴ However, possible somatic inactivation of the PCC/PGL predisposing genes by promoter methylation has so far not been fully determined. Cascon et al. reported unmethylated promoters for *SDHB*, *SDHD*, and *VHL* in 35 PCCs,¹⁵ and Dammann et al. described unmethylated *VHL* promoter in 25 PCCs.¹⁶ Later, Welander et al. reported unmethylated promoters for *KIF1Bβ*, *MAX*, *NF1*, *RET*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *TMEM127*, and *VHL* in 42 sporadic PCCs.¹¹ These three studies have employed methylation-specific PCR (MSP) for qualitative identification of hypermethylation. To unambiguously evaluate the gene promoter methylation status in these tumors, we employed quantitative Pyrosequencing in a large panel of PCCs and PGLs. Findings were subsequently compared with gene expression levels and evaluated against tumor and patient phenotypes.

Results

Increased promoter methylation density of *VHL* in PCC and PGL

CpG methylation density was quantified using Pyrosequencing for the promoter regions of 11 genes implicated in PCC/PGL (*EGLN1* [*PDH2*], *MAX*, *MEN1*, *NF1*, *RET*, *SDHAF2* [*SDH5*], *SDHB*, *SDHC*, *SDHD*, *TMEM127*, and *VHL*) in 96 tumors (83 PCCs, 13 PGLs) and 34 normal adrenal samples. For each sample and gene a methylation index (MetI) was defined as the mean methylation of all CpG dinucleotides covered. Overall, the methylation assessment for all genes except *VHL* showed no or very little methylation. With regards to *EGLN1*, *MAX*, *MEN1*, *NF1*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, and *TMEM127*, MetI values below 10% were obtained for all tumors and normal

adrenals (Table 1). One single CpG site (case PCC-56) within the *TMEM127* gene showed an increased methylation level of 15%. For the *RET* gene increased methylation was recorded in two PGLs (PGL-102 and PGL-113) with MetI values of 17% and 29%, respectively, while all other tumors and normal adrenals showed MetI below 10%.

By contrast, increased methylation was frequently observed for the *VHL* promoter (Fig. S1; Table 2). Comparing *VHL* methylation between sample groups (Fig. 1) revealed increased MetI in tumors as compared with normal adrenals (mean MetI 57% vs. 27%; *P* < 0.0001). DNA from 4 normal adrenal medullary samples (NAM-1–4) also showed low methylation levels (mean MetI = 31%) comparable to the normal adrenals. Altogether 72/96 (75%) of the tumors had a MetI > 50% while the MetI of the normal adrenals did not exceed 44% in any single case (Table 1). We further observed a difference in methylation level between specific CpG sites within the analyzed *VHL* promoter sequence (Table 2; Fig. 2; Fig. S1). This was particularly pronounced at CpG 1 and 2 (Chr3: 10 183 196 and Chr3: 10 183 198 respectively). Specifically, we observed a statistically significant (*P* < 0.0001, Table 2) higher methylation level in tumors as compared with normal adrenals at CpG 1 (mean methylation 80% vs. 37%), CpG 2 (72% vs. 36%), CpG 3 (45% vs. 19%), and CpG 4 (30% vs. 15%) (Fig. 2).

Increased *VHL* promoter methylation in malignant tumors and PGL

The results from *VHL* methylation analysis were compared with tumor and patient characteristics (Table S1). This revealed higher MetI values in malignant as compared with benign tumors (mean 63% vs. 55%; *P* = 0.009). Furthermore, we found higher MetI in PGLs vs. PCCs (mean 66% vs. 55%; *P* = 0.001). No significant correlations were found between MetI values and gender (*P* = 0.5), age at surgery (*P* = 0.65), tumor weight or size (*P* = 0.4), epinephrine levels (*P* = 0.14), norepinephrine levels (*P* = 0.53), dopamine levels (*P* = 0.7), constitutional *SDHB* mutations

Table 2. Results and correlations of methylation analyses of the *VHL* promoters

Mean methylation					
Parameter	CpG 1	CpG 2	CpG 3	CpG 4	MetI
Tumors vs. adrenals					
Tumors	79.9	72.1	44.2	30.0	56.6
Adrenals	37.3	36.4	18.9	15.3	27.0
<i>P</i> value	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
PGLs vs. PCCs					
PGLs	88.7	81.7	48.9	45.7	66.3
PCCs	78.6	70.6	43.5	27.5	55.0
<i>P</i> value	0.002*	0.003*	0.293	0.001*	0.001*
Malignant vs. benign					
Malignant	85.6	79.3	46.7	38.9	62.7
Benign	78.6	70.6	43.7	27.9	55.2
<i>P</i> value	0.097	0.011*	0.482	0.004*	0.009*
Methylation vs. gene expression					
<i>VHL</i> (assay 1) R =	-0.35	-0.31	-0.24	-0.26	-0.36
<i>P</i> value	<0.0001*	0.0003*	0.0071*	0.0027*	<0.0001*
Methylation vs. gene expression					
<i>VHL</i> (assay 2) R =	-0.23	-0.33	-0.10	-0.24	-0.27
<i>P</i> value	0.009*	0.0001*	0.252	0.006*	0.002*

*Statistically significant ($P < 0.05$); R, Pearson correlation coefficient.

($P = 0.1$) or clinical MEN 2A syndrome ($P = 0.31$). CpG 4 (Chr3: 10 183 220) showed the largest difference in methylation levels between PGLs and PCCs (mean methylation level 46% vs. 28%, $P < 0.001$) and between benign and malignant tumors (39% vs. 28%, $P = 0.004$) (Table 2).

Suppression of *VHL* gene expression

We studied the *VHL* gene expression in 74 tumors together with 13 normal adrenals using two individual *VHL* assays with qRT-PCR. To identify endogenous controls, we assessed 32 candidate housekeeping genes. We found 5 genes to be stably expressed among PCC, PGL and normal adrenal references. These include: *B2M*, *CASC3*, *MRPL19*, *PSMC4*, and *UBC* (Fig. S2). Significant changes were found in tumors as compared with normal adrenals. In general, the *VHL* gene expression was lower in tumors than in normal adrenals ($P < 0.0001$ for assay 1 and $P = 0.002$ for assay 2, Fig. 3). Furthermore, we found significantly lower *VHL* gene expression on PGLs as compared with PCCs for assay 2 ($P = 0.047$) but not for assay 1 ($P = 0.72$). Regarding tumor weight, we found significantly lower *VHL* expression in smaller tumors (Pearson correlation test $R = 0.24$, $P = 0.007$ with assay 2, $R = 0.11$, $P = 0.2$ with assay 1). Elevated norepinephrine levels were found significantly correlated with higher *VHL* gene expression ($P = 0.02$ for assay 1, $P = 0.15$ for assay 2). No significant correlations were found between *VHL* gene expression and gender ($P = 0.8$ for assay 1, $P = 0.5$ for assay 2), age at surgery ($P = 0.58$ for assay 1, $P = 0.73$ for assay 2), epinephrine levels ($P = 0.5$ for both assay 1 and 2), dopamine levels ($P = 0.9$ for assay 1, $P = 0.3$ for assay 2), constitutional *SDHB*

mutations ($P = 0.1$), or clinical MEN 2A syndrome ($P = 0.8$ for assay 1, $P = 0.6$ for assay 2). Furthermore, no significant correlation was found between *VHL* gene expression and malignancy ($P = 0.97$ for assay 1, $P = 0.63$ for assay 2).

Inverse correlation between *VHL* methylation status and mRNA expression

To further investigate the effect of *VHL* promoter methylation on its gene expression we compared MetIs for the promoter with *VHL* mRNA expression levels as determined by each of the two qRT-PCR assays. Pearson correlation analysis showed significant correlation ($R = 0.796$, $P < 0.0001$) between the two mRNA expression assays supporting that no methodological errors are present since the same gene was analyzed with dissimilar assays. Furthermore, MetI values were inversely correlated with *VHL* expression (Pearson correlation test $R = -0.359$, $P < 0.0001$ for assay 1 and $R = -0.232$, $P = 0.0077$ for assay 2). With regard to the individual CpGs, all sites showed inverse correlation between methylation level and *VHL* expression for assay 1 (Table 2).

Discussion

Using a quantitative approach we assessed the methylation status of the promoters of 11 PCC/PGL susceptibility genes in a large cohort of PCCs, PGLs and adrenal reference samples. We found significant hypermethylation of the *VHL* gene promoter in the tumor samples compared with normal adrenal references. *VHL* gene expression correlated inversely with the epigenetic status of the tumors, showing lower expression in tumors and

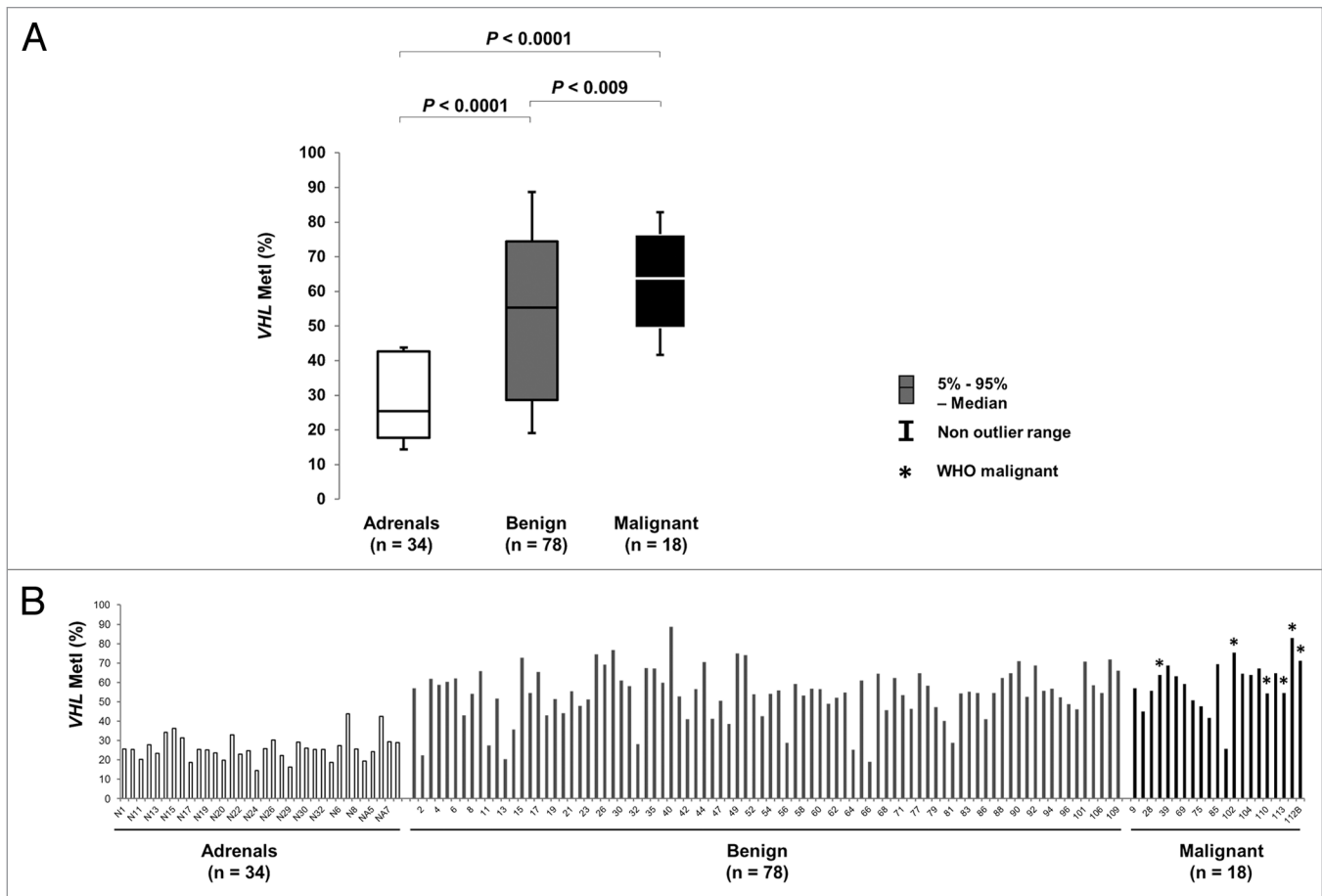


Figure 1. Comparison of *VHL* promoter methylation in normal adrenals and benign or malignant tumors. **(A)** Box plot showing Methylation index (MetI) for *VHL* in the different sample groups. **(B)** Column chart showing individual MetI for each sample. *WHO malignant cases.

higher expression in adrenal references. Given that gene silencing by promoter CpG methylation is an important epigenetic mechanism in tumorigenesis,¹⁷ our results indicate that *VHL* promoter hypermethylation could play a role in the development of PCC and PGL. Welander et al. have shown lower expression of the *VHL* gene in tumors with loss of one gene copy.¹¹ Other mechanisms for *VHL* inactivation could encompass involvement of microRNAs,¹⁸ mutations or, as our results indicate, promoter hypermethylation. Taken together our findings suggest that epigenetic *VHL* silencing is a frequently occurring and potentially disease-related event in PCC and PGL development.

In this study we observed very low methylation levels for all tested susceptibility genes except *VHL*. This is essentially in agreement with previous, non-quantitative publications discussing methylation analysis of PCC/PGL susceptibility genes.^{11,15,16} However, our observations contrast previously published conclusions concerning the *VHL* gene. This discrepancy may have several plausible explanations. Most important are the methodological differences; the previous studies that report unmethylated *VHL* gene promoters employed MSP^{11,15,16} and analyzed sequences located 127 nucleotides (6 CpG sites) downstream of the sequence analyzed in this study (Fig. 4). We found that the methylation density varies within the sequence of 4 CpG

dinucleotides covered by our *VHL* assay (Table 2; Fig. S1). It is therefore possible that the previously assessed CpG sites, located downstream of our assay, are indeed unmethylated—as surmised by Welander et al. and Dammann et al. However, a methylated *VHL* promoter has previously been found by Moore et al. in renal cell carcinomas employing primers targeting a sequence 302 nucleotides (38 CpG sites) downstream of the sequence analyzed in our study (Fig. 4).¹⁹ Previously, a genome-wide methylation analysis (Illumina Infinium HM27 DNA methylation assay) was undertaken for 145 PCCs and PGLs¹⁴ which did not report hypermethylation in the *VHL* promoter. This discrepancy with our data most likely stems from the fact that the CpG sites covered by the Illumina 27K chip are dissimilar to those analyzed in this study. The nearest location for any individual CpG site covered by the Illumina assay is found -1 kb downstream of the sequence assessed herein.

The increased *VHL* MetI levels in the tumors compared with normal adrenal references suggest that this is an important event in tumor development. A microarray study comparing rat PCCs with normal adrenal medullas revealed a number of differentially expressed genes, but did not identify *VHL* as one of them, indicating a difference between rat and human PCCs.²⁰ We also observed significantly increased MetI levels for *VHL*

in malignant as compared with benign cases and the *VHL* gene expression was inversely correlated with MetI values. However, the *VHL* gene expression did not show any statistically significant difference between malignant and benign cases. Likely, the variations in *VHL* gene expression may be present but can be too small to detect. Previously, gene expression profiling studies in human have not identified *VHL* as a differentially expressed gene between malignant and benign PCCs,²¹⁻²⁴ which is in agreement with our observations.

A CpG hypermethylator phenotype has been described for the PGL and *SDHB*^{12,13} as well as *SDHx*¹⁴ mutated entity. In this context it is of interest to note that the two cases with *RET* hypermethylation were malignant PGL harboring *SDHB* mutation (PGL-102 and PGL-113). Neither *VHL* promoter methylation nor *VHL* gene expression correlated with *SDHB* mutations and accompanying CpG island methylator phenotype (CIMP).¹³ The possible association to clinical VHL syndrome could not be evaluated as this was only observed in one patient.

In summary, our results show that the *VHL* gene promoter is hypermethylated in pheochromocytomas and paragangliomas, accompanied by stunted gene expression, a finding that highlights the importance of *VHL* inactivation in the etiology of these tumors.

Materials and Methods

Tumor samples and controls

Fresh frozen tissue material was obtained from the Biobank of the Karolinska University Hospital, Stockholm, Sweden. These samples had been collected with written or oral informed consent, as documented in patients' files, and sampling was approved by the local ethical committee. All tissues included were dissected and handled in accordance with established biobank protocols. The cohort included 96 tumors (83 PCCs, 13 PGLs; representative for the ratio of PCCs vs. abdominal PGLs operated at the Karolinska University Hospital) (Table S1). Thirty samples of non-tumorous adrenal tissues were used as normal adrenal references (Table S2). These samples were obtained in conjunction with renal or adrenal surgery and histopathologically verified as non-tumorous by two expert pathologists independently. In addition, we included 4 samples of commercially available DNA from normal adrenal medullas (NAM-1–4, Table S2) obtained

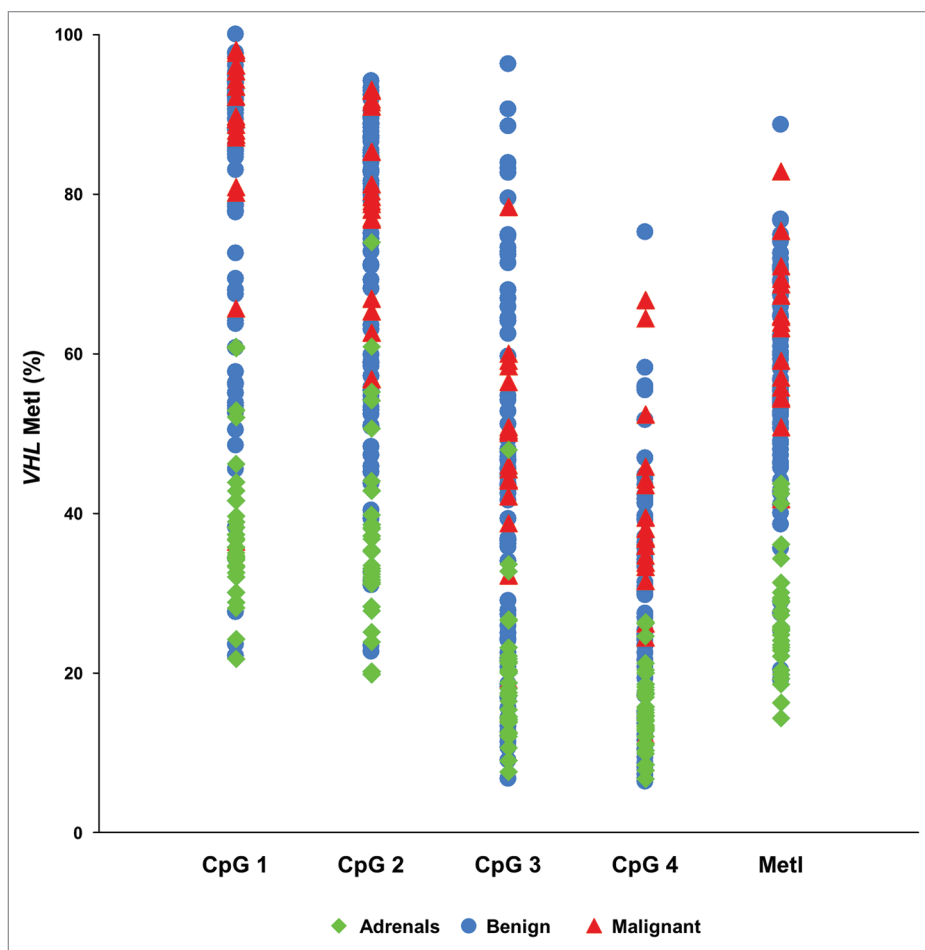


Figure 2. Methylation at each of the 4 individual CpGs of the *VHL* promoter in normal adrenals and tumors. The scatterplot shows Pyrosequencing values for each CpG and case analyzed together with the Methylation index (MetI). Normal adrenals, benign and malignant tumors are indicated with colors according to legend.

from autopsy of patients deceased from unrelated causes (Clinomics Biosciences Inc.).

Cases were characterized for gender, age of onset, hormonal status, existence of multifocal tumors, occurrence of multiple or bilateral tumors, tumor weight and tumor size, recurrence, duration of follow-up, patient outcome and time for metastasis. In addition, occurrence of constitutional *SDHB* mutations and clinical VHL, MEN 2A, and NF1 syndrome is given in Table S1. In the current paper malignant cases were identified based on metastasis and/or extensive local invasion according to the Armed Forces Institute of Pathology (AFIP) guidelines,²⁵ due to higher sensitivity in detecting potentially deleterious tumors (herein referred to as malignant). Additionally, we have given the classification according to the World Health Organization (WHO) guidelines¹ (referred to as WHO malignant), since these are also widely accepted for the classification of these tumors. The WHO criteria base malignancy solely on the presence of distant metastases.¹ A small representative piece was cut from 115/130 (88%) of all analyzed samples. The piece was thawed and fixed in formaldehyde solution, embedded in paraffin, sectioned and

stained with Haematoxylin and Eosin. Tumor or normal cell content was subsequently determined by two expert pathologists, independently revealing representative tumor or normal cell content of more than 70% in 92% of the included cases (data not shown). Cases that revealed tumor or normal cell content <50% were excluded from the study.

Preparation of DNA and RNA

Extraction of genomic DNA was performed using DNeasy Blood and Tissue DNA isolation kit (QIAGEN AB). Quantification and initial quality check of the extracted DNA was performed using a NanoDrop A100 (Thermo Scientific). EpiTect Bisulphite conversion kit was used for bisulphite conversion of 500 ng of genomic DNA from all tumors and normal samples (QIAGEN AB). The method is described in detail in a preceding publication.²⁶

Extraction of total RNA was performed using the *mirVana* miRNA Isolation Kit (Ambion, Life Technologies, Applied Biosystems) with no changes to the original protocol. RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent) with the RNA Nano 6000 chips and reagents (Agilent) (Tables S1 and S2).

Pyrosequencing and data analysis

Pyrosequencing was performed to assess the methylation level of PCC and PGL susceptibility genes in 96 tumors (83 PCCs, 13 PGLs) and 34 adrenal/adrenal medullary reference samples. The assays for *EGLN1*, *MAX*, *MEN1*, *NF1*, *RET*, *SDHAF2*, *TMEM127*, and *VHL* are commercially available (PyroMark Assay Database, Geneglobe, QIAGEN). The *SDHB*, *SDHC* and *SDHD* assays were ad hoc-designed using PyroMark Assay Design software version 2.0 (QIAGEN). Both newly designed and commercially available assays were extensively tested for assay bias as previously described¹³ (results not shown). Assay specifications and sequences are summarized in Table S3. We also made several attempts at designing primers for *SDHA*, and for the *VHL* region corresponding to the target sites in an article by Dammann et al.,¹⁶ however these assays did not produce reliable results and were therefore excluded.

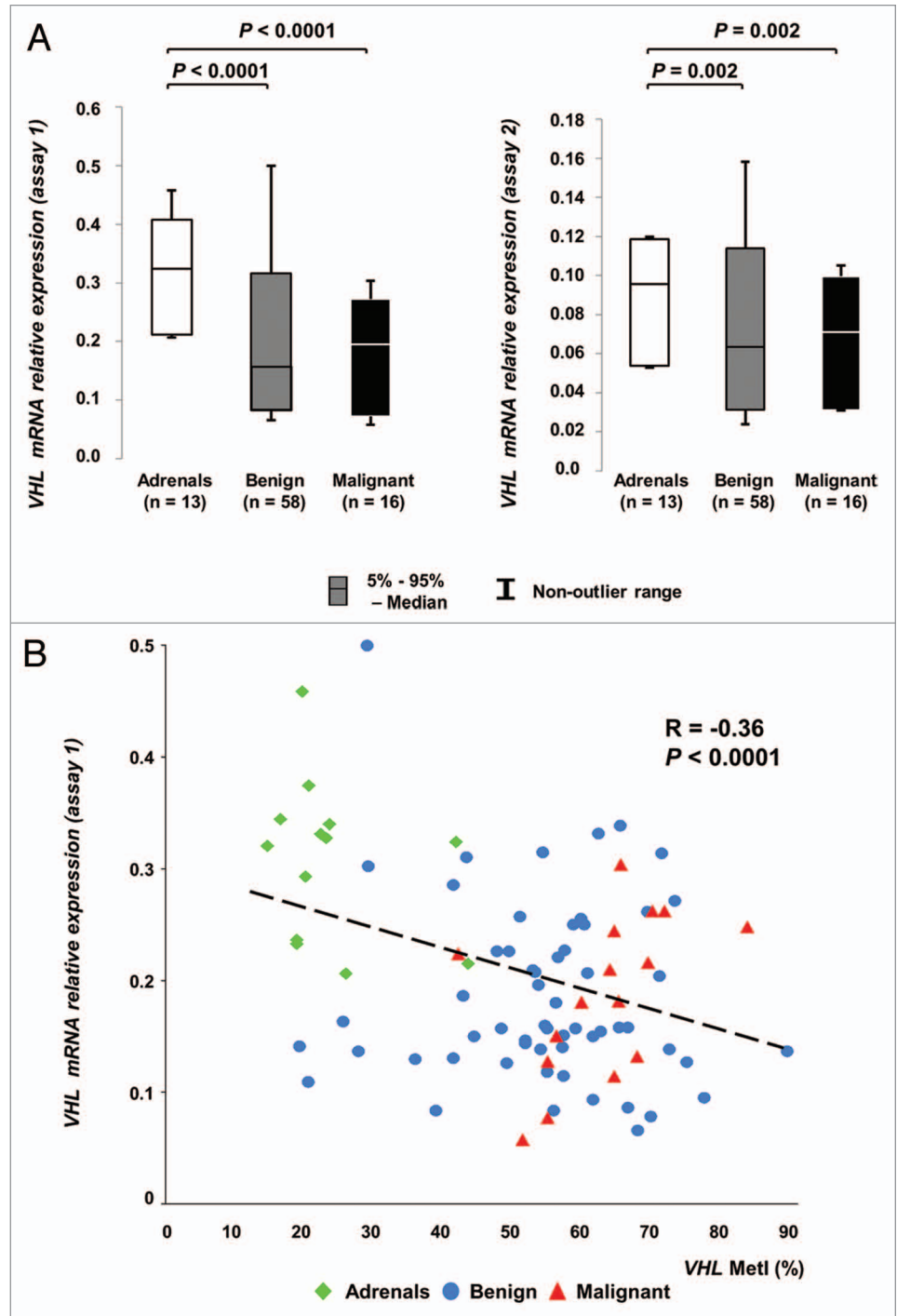


Figure 3. Comparison of promoter methylation density and gene expression for *VHL*. (A) Boxplots showing relative *VHL* mRNA expression (y-axis) in the sample groups (x-axis) determined by qRT-PCR using two different assays. (B) Scatterplot including trend line showing Met1 values (x-axis) and relative mRNA expression levels determined by assay 1 (y-axis).

Pyrosequencing has been described in detail in a previous publication.²⁷ In short, pre-made master mix—including DNA polymerase from QIAGEN—was used to amplify 1 μ l of bisulphite-treated DNA in a PCR reaction, using the following conditions: 95 $^{\circ}$ C for 15 min, followed by 45 cycles of: (95 $^{\circ}$ C 30 s,

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/26686

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