DNA Methylation Profiling in Pheochromocytoma and Paraganglioma Reveals Diagnostic and Prognostic Markers

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Abstract

Purpose: Pheochromocytoma and paraganglioma (PPGL) are rare neuroendocrine tumors, associated with highly variable postoperative evolution. The scarcity of reliable PPGL prognostic markers continues to complicate patient management. In this study, we explored genome-wide DNA methylation patterns in the context of PPGL malignancy to identify novel prognostic markers.

Experimental Design: We retrospectively investigated DNA methylation patterns in PPGL with and without metastases using high-throughput DNA methylation profiling data (Illumina 27K) from two large, well-characterized discovery (n = 123; 24 metastatic) and primary validation (n = 154; 24 metastatic) series. Additional validation of candidate CpGs was performed by bisulfite pyrosequencing in a second independent set of 33 paraffin-embedded PPGLs (19 metastatic).

Results: Of the initial 86 candidate CpGs, we successfully replicated 52 (47 genes), associated with metastatic PPGL. Of these, 48 CpGs showed significant associations with time to progression even after correcting for SDHB genotype, suggesting their value as prognostic markers independent of genetic background. Hypermethylation of RDBP (negative elongation factor complex member E) in metastatic tumors was further validated by bisulfite pyrosequencing [Δβmethastatic-benign = 0.29, P = 0.003; HR, 1.4; 95% confidence interval (CI), 1.1–2.0; P = 0.018] and may alter transcriptional networks involving (RERG, GPX3, and PDZK1) apoptosis, invasion, and maintenance of DNA integrity.

Conclusion: This is the first large-scale study of DNA methylation in metastatic PPGL that identifies and validates prognostic markers, which could be used for stratifying patients according to risk of developing metastasis. Of the three CpGs selected for further validation, one (RDBP) was clearly confirmed and could be used for stratifying patients according to the risk of developing metastases. Clin Cancer Res; 21(13):3020–30. © 2015 AACR.

Introduction

Pheochromocytoma and paraganglioma (PPGL) are rare neuroendocrine tumors that arise from the adrenal medulla and paraganglial system, respectively. PPGLs can arise as part of hereditary syndromes associated with germline mutations in at least 15 genes and have been extensively discussed elsewhere (1, 2). Extensive clinical and genetic characterization over the last 25 years has greatly improved our knowledge about the genetic basis underlying PPGLs. Fortunately, application of high-throughput
Translational Relevance

Pheochromocytoma and paraganglioma (PPGL) are rare tumors that often present highly variable, postoperative outcomes. Current therapeutic options for metastatic PPGL are very limited. Therefore, novel prognostic markers for metastatic PPGL are urgently needed. In this study, we investigated, identified, and validated novel prognostic and predictive markers for PPGL that present metastases using whole-genome DNA methylation profiling data from two large, well-characterized discovery and primary validation series of tumors. Even after correcting for SDHB genotype, 48 of these CpGs showed significant associations with time to progression, indicating potential utility as novel molecular predictive markers. Our findings suggested that aberrant DNA methylation might affect nervous system development and transcriptional regulation networks in PPGL with metastases, providing potential clues for future therapeutic strategies. Specifically, these analyses indicated RDBP hypermethylation in metastatic tumors, which we validated in another second independent series, disrupting the expression of various key molecules that we also observed in transcriptomic analyses.

In this study, we explored DNA methylation patterns in metastatic PPGL using two large, well-characterized cohorts of PPGL collected through a multi-institutional collaboration within the European Network for the Study of Adrenal Tumors (ENS@T). Candidate CpGs associated with metastatic PPGL were identified using whole-genome DNA methylation profiles obtained from a discovery series containing 24 metastatic (4 SDHB) PPGLs. These data were replicated for 52 of these candidate CpGs in a first validation series, which comprised whole-genome DNA methylation data (27K Illumina) containing 154 PPGL, including 24 metastatic tumors (10 SDHB). Interestingly, significant associations with time to progression (TTP) were found for a large proportion of these CpGs. Among these CpGs, hypermethylation of the RDBP (negative elongation factor complex member E) gene in metastatic PPGLs was further validated in a third series by bisulfite pyrosequencing [ΔHazard ratio (HR) metastatic–benign = 0.29; P = 0.003; 95% confidence interval (CI), 1.1–2.0; P = 0.018] of 33 formalin-fixed, paraffin-embedded (FFPE) tissues. Bioinformatics analyses suggested that RDBP hypermethylation might contribute to metastatic disease by altering transcriptional networks, including those involved in response to apoptotic stimuli, invasion, and maintenance of DNA integrity. Our results suggest that these CpGs may represent potential markers of malignancy and also serve as prognostic predictors independent of genetic background.

Materials and Methods

Patient samples

According to standard protocols, all patients underwent tumor resective surgery 4 to 6 weeks after diagnosis of PPGL. All fresh-frozen tissues were immediately frozen in liquid nitrogen and stored at −80°C. PPGL was confirmed by pathologic examination. All samples included in this study contained at least 85% tumor cells. A PPGL was considered metastatic when the patient presented metastases at non-chromaffin sites distant from the primary tumor (Supplementary Table S1).

For this retrospective study, a total of 310 tumors were obtained from patients with confirmed PPGL diagnosis, recruited through collaborating centers of the European Network for the Study of Adrenal Tumors (ENS@T) according to the Reporting Recommendations for Tumor Marker prognostic Studies (REMARK) guidelines (12). Discovery series (n = 123) and validation series 2 (n = 33) samples were collected from 1989 to 2012 through seven institutions: Spanish National Cancer Research Centre (Madrid, Spain); Erasmus MC Cancer Institute (Rotterdam, the Netherlands); Ludwig-Maximilians-University of Munich (Munich, Germany); Radboud University Medical Centre (Nijmegen, the Netherlands); Veneto Institute of Oncology (Padua, Italy); Technische Universität Dresden (Dresden, Germany); and University of Florence (Florence, Italy). Validation series 1 samples (n = 154) were collected from patients recruited through the COMETE (Cortico et Médullosurréale: les Tumeurs Endocriques) network from 1993 to 2008 (2). All patients provided written informed consent for the collection of samples and subsequent analyses. Ethical approval for the study was obtained from each institutional review board from all collaborating centers.

Median age and gender ratio (female: male) were 44 years and 1.28, respectively. There were no statistically significant differences among median age distribution and gender ratio between series (Mann–Whitney U associated P > 0.05). These 310 tumors...
represent three series: discovery series (DS) and validation series (VS1 and VS2). The DS was composed of 123 fresh-frozen PPGL specimens (24 metastatic). Genetic screening for DS samples was performed as previously described (3, 13, 14). The 99 PPGLs without metastasis contained 22 VHL, 26 RET, 8 NF1, 4 SDHB, 2 SDHD, 4 EPAS1, 2 MAX, 2 HRAS, and 1 TMEM127 mutations, and 28 tumors ("wild-type" or WT) had no mutations in known susceptibility genes. The 24 metastatic tumors were mostly WT (n = 17) with the rest being 4 SDHB-, 2 MAX-, and 1 VHL-related PPGLs.

DNA methylation data from another large cohort (VS1) of PPGLs was obtained from Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/gds), under accession number GSE39198 (2). VS1 was expanded with 10 additional metastatic specimens (previously unpublished), also provided by the COMETE network. VS1 contained a total of 154 PPGLs. Of these, 24 were metastatic tumors and included 10 SDHB-, 5 VHL-, 2 NF1-, and 1 FH-related PPGLs, as well as 6 WT tumors. VS1 samples were collected prospectively by the COMETE network, and genetic screening was performed as previously described (2). Ethical approval for the study was obtained from the local institutional review board [Comité de Protection des Personnes (CPP) Ile de France III, June 2012]. Written informed consent for the sample collection and subsequent analyses was obtained from all patients (2).

For further validation, we compiled an additional independent series (VS2) of FFPE PPGLs (n = 33; 19 metastatic) through ENs@T. Like DS and VS1 cohorts, VS2 was composed of only pheochromocytomas and sympathetic paragangliomas, and contained no parasympathetic paragangliomas. Of these 33 PPGLs, 19 were metastatic (16 WT, 1 SDHA, 1 HRAS, and 1 VHL) and 14 samples were nonmetastatic (3 SDHB, 2 VHL, 1 HRAS, and 8 WT). Tumors from Erasmus MC University Medical Center were assessed anonymously according to the Proper Secondary Use of Human Tissue code established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

A summary of clinical features for DS, VS1, and VS2 PPGLs is shown in Table 1 (more detailed clinical information is provided in Supplementary Table S1).

### DNA extraction and purification and DNA methylation assay

As for VS1 samples, DNA from DS and VS2 samples was extracted and purified using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol (2). Bisulfite conversion of DNA was performed using the EZ DNA Methylation Kit (Zymo Research) following the manufacturer’s recommendations. Genome-wide DNA methylation was assayed using the Illumina Infinium HumanMethylation 27K Platform (Illumina) at the Spanish, "Centro Nacional de Genotipo (CEGEN-ISCIII)" (www.cegen.org), as previously described (15).

### Data processing

The Illumina 27K array interrogates 27,578 CpGs. \( \beta \) values for each interrogated CpG were assigned using the Genome Studio Methylation module. To correct for the heteroskedasticity observed for \( \beta \) values, \( \beta \) values were transformed to M-values, defined as \( \log_2 \) (methylation probe intensity/unmethylated probe intensity). Negative M values indicate less than 50% methylation and positive M values indicate more than 50% methylation (16). Thus, M values were used for all statistical analysis (supervised and TTP analyses), whereas \( \beta \) values were used for biologic interpretation (e.g., difference in methylation; ref. 16).

### Data analysis and statistical methods

#### All unsupervised and supervised analyses were performed using "R." The top 500 probes with the highest variance across all samples were used for unsupervised analysis. The "Consensus-ClusterPlus" package was used for consensus cluster analysis (17).

#### For all statistical analyses performed involving multiple testing, \( P \) values were adjusted accordingly by the Benjamini and Hochberg method (q-values; ref. 18). All supervised analyses were performed using the "limma" package and were limited to experimental groups with at least three samples. All samples in DS (n = 123) and VS1 (n = 154; Supplementary Table S1) were included in supervised analyses. Only significantly methylated CpGs (\( q < 0.10 \) and \( \Delta \beta > 0.2 \)) were considered for subsequent analyses.

#### Comparisons between the different PPGL genetic groups were performed always relative to the SDHB group to facilitate inter-group comparisons.

First, candidate CpGs associated with metastatic PPGL were identified by supervised analysis comparing DS samples with and without metastasis (\( q < 0.10 \) and \( \Delta \beta > 0.2 \)). Candidate CpGs were further distilled filtering out those probes not present in at least 50% of metastatic PPGLs. To refine selection of candidate CpGs, DNA methylation patterns of candidate CpGs were assessed in VS1 only. Those successfully replicated CpGs were considered for further analyses. SDHB PPGLs have recently shown to have a CpG island hypermethylator phenotype (high-CIMP) with a previously defined signature of 298 hypermethylated CpGs (2). Thus, we further filtered the data for these probes. Those CpGs meeting all the above criteria were considered as candidate CpG markers associated with metastatic PPGL.

### Table 1. Clinical summary for methylation study tumors

<table>
<thead>
<tr>
<th></th>
<th>DS</th>
<th>VS1</th>
<th>VS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>#</td>
<td>Ben*</td>
<td>Met</td>
<td>Ben*</td>
</tr>
<tr>
<td>VHL</td>
<td>23</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>RET</td>
<td>26</td>
<td>26</td>
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<tr>
<td>NF1</td>
<td>8</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>SDHA</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SDHB</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SDHC</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SDHD</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>FH</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>EPAS1</td>
<td>4</td>
<td>4</td>
<td>—</td>
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<td>MAX</td>
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<td>4</td>
<td>4</td>
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<td>HRAS</td>
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<td>2</td>
<td>—</td>
</tr>
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<td>TMEM127</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>WT</td>
<td>45</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td>N(^\circ) PPGL</td>
<td>125</td>
<td>99</td>
<td>24</td>
</tr>
</tbody>
</table>

*Total samples.

*Tumors without metastases.

†Tumors with metastases. Tumors were considered metastatic when patients presented metastasis in sites normally devoid of chromaffin tissue (e.g., lung, liver, bone, lymph nodes), either synchronous or metachronous. Diagnosis of metastasis was based on imaging evidence or pathologic confirmation.

‡Normal adrenal medulla (not included in analysis).
documented detection of metastases (metastases delay). Patients without documented evidence of metastases were censored at the date of last follow-up. Only samples from patients presenting metachronous metastases were included in survival analyses, whereas samples from patients with synchronous metastases, defined as those presenting at diagnosis or surgery (event time = 0), were excluded from these analyses. As DS and VS1 data were generated with the same platform, we pooled samples to form a larger collection of tumors to permit TTP analyses. After filtering out samples from patients with synchronous metastases (n = 25), as well as those samples without complete follow-up (n = 19), there remained 233 tumors including 23 metastatic PPGLs (events). The final model was adjusted for SDHB mutation, series origin of tumors, and multiple testing as described above (18). For Kaplan–Meier plots, methylation M values were converted to binary variables using the median as the cutoff value. These variables were defined as 0 (hypomethylated) for those values less than the median cutoff, whereas values greater than the cutoff were considered as 1 (hypermethylated). The association between methylation at individual CpGs and TTP was estimated using the method of Kaplan and Meier and assessed using the log-rank test. Kaplan–Meier curves and analyses were performed using SPSS version 17 (SPSS Inc.).

Venn diagram analyses were performed with the "Vennerable" package (http://vennerable.sourceforge.net/), and circos plots were generated using the "circlize" and "RCircos" package. Pathway analysis was performed using the PantherDB classification system (http://www.pantherdb.org/) and DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp).

Bisulfite pyrosequencing

Among those candidate CpGs associated with metastatic PPGL, three were selected for further validation by bisulfite pyrosequencing in VS2 (33 FFPE PPGLs). These CpGs were selected because they showed highly different methylation levels and because of the reported biologic functions of the corresponding genes.

Bisulfite modification of DNA was performed as described above. Primers for PCR amplification and sequencing were designed with PyroMark assay design version 2.0.01.15 software. Primer sequences were designed to hybridize with CpG-free sites to ensure methylation-independence amplification (Supplementary Table S2). PCRs were performed with biotinylated primers to convert the PCR product to single-stranded DNA templates with the Vacuum Prep Tool. After PCR amplification, pyrosequencing reactions and methylation quantification were performed using PyroMark Q24 reagents, equipment, and software according to manufacturer’s instructions (Qiagen). Quantification of DNA methylation for a given CpG by bisulfite pyrosequencing is given as the percentage of 5-methylcytosine representative of the C-to-T ratio (100 × β value). Significance was assessed by Student t test. TTP was calculated in FFPE samples by Cox regression for RDBP as continuous variables (M values), as described above.

GEO datasets

Gene expression profiling data for 99 PPGLs, containing 8 with and 91 without metastasis, were obtained through gene expression omnibus (GEO) under GSE19422 and GSE51087. Gene expression profiling data for T47D breast cancer cells with and without RDBP knockout was accessed through GEO (GSE19940). As previously described, downloaded gene expression profiles were quantile normalized and antigenomic probes were used as background probes (3, 19, 20).

Results

DNA methylation profiling in DS and VS1

DNA methylation data for DS tumors were deposited on GEO under the GEO accession number GSE62231 and the 10 additional metastatic PPGLs of the VS1 tumors from the COMETE network under the accession number GSE43298. Probes on the X or Y chromosome (n = 1,085 or n = 7, respectively), as well as unreliable probes not detected with P > 0.01 in more than 5% of samples (n = 1029), were removed. The remaining 25,457 probes were used for subsequent analyses. After preprocessing, we obtained β and M values of these CpGs for DS PPGLs.

Unsupervised and supervised analyses of PPGL DNA methylation profiles

Unsupervised hierarchical cluster analysis in DS, split samples into two main clusters, C1 and C2 (Fig. 1A). C1, enriched with VHL- and SDHx-related tumors, was subdivided into 2 subclusters (C1A and C1B). C1A contained seven of eight SDHB- and all SDHD-related and 5 WT PPGLs. In total, C1A was composed of 15 tumors, 6 were metastatic. C1B contained 22 VHL-, 1 SDHB-, 6 RET-, 3 NF1-, and 3 EPAS1-related and 11 WT tumors. C1B contained 37 PPGLs, 9 with metastases. In addition to 20 RET-related tumors, C2 contained 5 NF1-, 2 HRAS-, 1 TMEM127-, 1 VHL-, and 1 EPAS1-related PPGLs and 28 WT specimens. Nine of 62 tumors in C2 were metastatic.

K-means consensus clustering was performed to verify hierarchical cluster analysis. According to this analysis, the optimal classification defined 3 subgroups, shown in Fig. 1B. Cluster memberships for hierarchical cluster and consensus cluster analyses results were highly similar with a 93% (114 of 123) agreement between the two. Cluster memberships for DS are provided in Supplementary Table S1.

We generated a circos plot (Fig. 1C) depicting methylation levels for six genetic classes (SDHB, VHL, EPAS1, RET, NF1, and MAX). Figure 1C and D show varying degrees of methylation among the six groups. SDHB-related tumors had highest levels of DNA methylation. VHL- and EPAS1-related PPGLs showed intermediate levels, whereas RET-, NF1-, and MAX-related tumors had the lowest levels of DNA methylation. The difference between genotypes was significant (ANOVA; P = 0.001). These results verify that SDHB-related tumors display a CpG island methylator phenotype (high-CIMP).

Initial comparisons between the different genetic groups and SDHB-related tumors in DS identified numerous hypermethylated and hypomethylated CpGs (Table 2). To validate the above DNA methylation patterns associated with each genetic background, the same analyses were performed in VS1. These analyses were performed for VHL-, RET-, NF1-, MAX-, and SDHB-associated tumors in VS1, but not for EPAS1-related samples, as none were available in VS1 (Table 2). To further determine genotype specific CpGs, as well as those common among all genotypes, we performed Venn diagram analysis with these confirmed probes (Fig. 1E). As expected, CpGs in SDHB-related tumors were almost all hypermethylated, whereas the other experimental groups showed a higher proportion of hypomethylated CpGs.
We performed pathway analysis using differentially methylated genes for each intergroup comparisons of PPGLs. Comparisons versus SDHB-associated PPGLs showed enrichment in pathways implicated in tumorigenesis, such as "pathways in cancer," "MAPK signaling pathway," "p53 signaling pathways," "ECM-receptor interaction," "focal adhesion," and "apoptosis." Well-known TSGs and mediators of apoptosis were among the hypermethylated genes in SDHB-related tumors (Fig. 1C). The most overrepresented pathways among VHL-, RET-, NF1-, and MAX-related tumors relative to SDHB-associated PPGLs were "WNT
Table 2. Differentially methylated CpGs for PPGL experimental group comparisons

<table>
<thead>
<tr>
<th>Comparison</th>
<th>CpGs in DSa</th>
<th>Replated in VS1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL vs. SDHB</td>
<td>143</td>
<td>1,265</td>
</tr>
<tr>
<td>RET vs. SDHB</td>
<td>4</td>
<td>172</td>
</tr>
<tr>
<td>NFI vs. SDHB</td>
<td>12</td>
<td>1,476</td>
</tr>
<tr>
<td>MAX vs. SDHB</td>
<td>8</td>
<td>782</td>
</tr>
</tbody>
</table>

*aCpGs initially identified in DS.  
*bCpGs successfully replicated in VS1.

DNA methylation profiling in PPGL: Novel Prognostic Markers

**Gene expression profiling of RDBP knockout**

To identify potential RDBP target genes, we downloaded gene expression data for T47D breast cancer cells with and without RDBP knockout from GEO (GSE19940; ref. 19). Upon RDBP knockout, 700 genes were differentially expressed (\(q < 0.05\) and \(\log_{2} \text{fold} > 1.0\)). Among these, many genes were downregulated, such as PDZKI (PDZ domain containing 1), RERG (Ras-like, estrogen-regulated, growth inhibitor), GPX3 (glutathione peroxidase 3), TIMP1 (tissue inhibitor of metalloproteinase 1), and CDKN2C (cyclin-dependent kinase 4 inhibitor C). We also observed a mild but significant induction of JUNB upon RDBP knockout in the T47D data. Using available gene expression data, we observed decreased expression of PDZKI, RERG, and GPX3 in metastatic PPGL. Unfortunately, we could not evaluate RDBP expression in this dataset, as it was not represented.

**Discussion**

DNA methylation is involved in long-term gene expression programming of cell-type identity and thus provides a novel reservoir for prognostic markers. Methylation patterns have already proven valuable as prognostic markers in other neoplasms, such as colon and breast cancers. Therefore, it is reasonable to consider that metastatic PPGLs might have unique profiles of DNA methylation that may differentiate them from those without metastasis and also might predict clinical outcome. Early attempts to uncover epigenetic prognostic markers in PPGL generally focused on TSG hypermethylation (10, 11). Currently, gDNA methylation patterns can be interrogated using high-throughput technologies. In fact, global hypermethylation was recently described in SDHx-related PPGLs caused by Kreb’s cycle dysfunction (2, 8). However, these data still are limited and their prognostic value is unclear.

In the present work, whole-genome DNA methylation profiles were obtained using the 27K platform (Illumina). Although the newer 450K platform has replaced the 27K platform, evidences indicate that in terms of general performance both produce consistent and comparable results as previously demonstrated (2). Our results verified that PPGL methylation patterns are strongly influenced by genetic background, exemplified by hierarchical cluster analysis showing two clusters: a "pseudohypoxic" cluster (VHL, SDHx, and EPAS1) and the other containing classical cluster 2 (RET, NF1, TMEM127, and MAX) PPGLs. In addition, unsupervised analysis of DNA methylation profiles proved capable of differentiating SDHx-related PPGLs from VHL/EPAS1- and RET/NF1/MAX-associated tumors. However, hierarchical cluster analysis of DNA methylation profiles was unable to further resolve cluster 2 samples. The two HRAS-related tumors were grouped along with cluster 2 tumors. This was not surprising as HRAS-related and other cluster 2 tumors (RET, NF1, TMEM127, and MAX) converge on a common molecular mechanism characterized by activated MAPK signaling.

DNA methylation differed significantly among PPGL groups (\(P < 0.0001\)). SDHB-related tumors showed highest levels of DNA methylation, whereas RET-, NF1-, TMEM127-, and MAX-related PPGLs showed the lowest levels. VHL- and EPAS1-related tumors
had intermediate levels of global DNA methylation with slightly higher levels in EPAS1-associated PPGCs. Our results showed the vast majority of CpGs in SDHB-related PPGCs were hypermethylated and verify previous reports suggesting high-CIMP (2, 8). A wide variety of human neoplasms have been described with high-CIMP (21–23). Prognosis associated with high-CIMP is cell-type-dependent. In glioblastoma and pediatric T-cell acute lymphoblastic leukemia, high-CIMP is associated with a more favorable prognosis, whereas poor prognosis has been reported for neuroblastoma and PPGC (2, 21–23). SDHx PPGCs, particularly SDHB-related have a higher risk to develop metastatic disease (24).

Recently, FH mutations were described in association with high-risk of metastatic disease (25). The Letouzé and colleagues study included 14 metastatic specimens of which one had an FH
DNA methylation profiling in PPGL: novel prognostic markers.

DNA methylation patterns associated with metastatic CpGs. A, Circos plot showing 52 validated. Outermost track provides ideogram for all chromosomes, except chromosomes 14, 18, 21, 22, X, and Y. Heatmaps show methylation levels for 52 confirmed CpGs associated with metastatic PPGL: (a) metastatic tumors in DS; (b) tumors without metastases in DS; (c) metastatic tumors in VS1; and (d) tumors without metastases in VS1. Green dots indicate gene with functions in nervous system development, whereas genes involved with transcriptional regulation at gene promoters are indicated by orange dots. Hypermethylated CpGs are indicated in red, whereas hypomethylated CpGs are indicated in blue. B-D, Kaplan-Meier survival curves for select CpGs associated with worse TTP (n = 233, 23 events). Red line indicates hypermethylated tumors and blue line represents hypomethylated tumors. The HR and associated q value (P value adjusted for SDHB status and series and multiple testing) represented were considering methylation levels as continuous variables. B, RDBP_cq06351503: HR, 3.0 (95% CI, 2.0–4.4); q = 0.001. Log-rank test P = 6.3 × 10^−5. Hypermethylated (n = 117, 21 events); hypomethylated (n = 116, 2 events); median methylation: M = 0.93, b = 0.34. C, HDAC11_cq05446471: HR, 1.5 (95% CI, 1.1–2.0); q = 0.047. Log-rank test P = 0.007. Hypermethylated (n = 118, 20 events), hypomethylated (n = 115, 3 events); median methylation: M = 0.29, b = 0.45. D, CYFIP2_cq00986320: HR, 2.0 (95% CI, 1.3–2.9); q = 0.006. Log-rank test P = 0.002. Hypermethylated (n = 118, 20 events), hypomethylated (n = 115, 3 events); median methylation: M = 0.77, b = 0.63.

Supervised analyses comparing VHL-, RET-, NF1-, and MAX-related tumors revealed specific DNA methylation patterns. We observed hypermethylation of putative TSGs in mutation and six had SDHB mutations. Severe derangement of metabolic homeostasis caused by mutations in FH, SDHx, and isocitrate dehydrogenase genes leads to global hypermethylation due to inhibition of α-ketoglutarate–dependent dioxygenases, including histone demethylases and TET 5-methylcytosine dioxygenases (2, 8, 26). However, it is likely that the reported association between poor prognosis and high-CIMP in PPGLs is linked to SDHB and FH mutations, as these are themselves associated with worse outcome. Our hierarchical cluster analysis, showing metastatic PPGLs distributed throughout the dendrogram, provided further evidence indicating that global hypermethylation, whereas undoubtedly relevant, is not necessarily sufficient to give rise to metastasis.
**SDHB**-related tumors, consistent with high-CIMP in other tumors (9). Also, many pathways potentially affected by DNA methylation in these tumors have important roles in tumorigenesis, such as pathways in cancer, "MAPK signaling pathway," and "p53 signaling pathway." It is well-known that p53 is essential for cellular responses to DNA damage, and in the great majority of human neoplasms, the p53 network is disrupted (27). It is tempting to speculate that hypermethylation of these TSGs in **SDHB**-related tumors could prevent induction of apoptosis by severe mitochondrial dysfunction caused by **SDH** deficiency.

Differential methylation of PNMT was of particular interest due to its role as a marker of chromaffin cell differentiation and in steroid-induced catecholamine biosynthesis (28). Our results verified PNMT hypermethylation previously reported in **SDH**-related PPGLs (2). PNMT hypomethylation in RET- and NF1- and hemimethylation in VHL-related PPGLs is highly consistent their associated neurochemical phenotypes (2, 20, 29). However, this model does not explain why other "cluster 1" tumors do not display an adrenergic phenotype, even when PNMT is hemimethylated. Also, this does not explain why MAX-associated tumors, showing PNMT hypomethylation comparable to RET and NF1 tumors, do not display a predominantly adrenergic phenotype. In this context, Qin and colleagues (2014) provided evidence that addressed these paradoxes in other "cluster 1" tumors, do not display a predominantly adrenergic phenotype. In this context, Qin and colleagues (2014) provided evidence that addressed these paradoxes in other "cluster 1" tumors characterized by both EPAS1 expression and stabilization of HIF protein (20). In that study, the authors showed that EPAS1 completely blocked effects of steroid-induced PNMT expression in PC12 cells (29). Altogether this provides a rational explanation why all "cluster 1" tumors characterized by both a high-CIMP phenotype (e.g., VHL and EPAS1), do not produce significant epinephrine, as well as the unexpected nonadrenergic phenotype presented by MAX tumors.

Hypermethylation of MEG3 was observed in MAX-related PPGLs. MEG3 is a noncoding TSG located on chromosome 14q32 that has been shown to interact with p53 (30). Downregulation of MEG3 in MAX-related PPGLs has been previously reported (14), and here we provide further evidence indicating that MEG3 downregulation is caused by aberrant hypermethylation due to a uniparental (paternal) disomy or partial or entire chromosome 14 deletions.

Although not selected for validation by pyrosequencing, it merits mention that other CpGs associated with metastatic PPGLs were not only identified in the DS but also replicated in VS1. In addition, none were contained in the **SDH**-related list (2), which suggested these CpGs are indeed associated with metastatic PPGLs and not genetic background. Not to mention, many also showed a significant association with TTP even after adjusting for **SDH** mutation.

For instance, **HDAC11** expression has been described to correlate inversely with proliferative status in nontransformed fibroblasts (31). Also among other candidate CpGs, hypermethylation of the RASSF1 promoter has been previously described in association with metastatic PPGL and neuroblastoma (2, 10, 11). The probe described by Letouzé and colleagues differed from the one identified in the present study, as these CpGs mapped to different genomic loci, explaining why it was not removed when we filtered for **SDH**-associated CpGs (2).

Bioinformatics analyses indicated these CpGs could be involved in nervous system development and regulation of transcription at RNA polymerase II promoters. Initially described as an oncogene, NTRK1 is primarily expressed in sensory and sympathetic neurons and considered a critical receptor promoting neuronal survival in the presence of NGF. However, recent evidence indicates that NTRK1 acts as a "dependence receptor," as expression of NTRK1 by itself was described to cause neuronal cell death and this activity was prevented by addition of NGF (32, 33). It is widely accepted that PPGL arises through a common mechanism that impedes apoptosis of sympathoadrenal precursors when NGF becomes limiting, which involves NTRK1 (34). Also with roles in nervous system development, CYFIP2 is a p53-inducible gene that leads to caspase activation and apoptosis (35, 36). Similarly, CYFIP2 has been shown to negatively modulate survival of colon cancer cells (37). Epigenetic inactivation of SPOCK2 gene is involved in malignant transformation of ovarian endometriosis (38, 39). Interestingly, **PG**1 and **BCL**9L, hypermethylated in metastatic PPGL, participate in the nuclear β-catenin/TCF complex (35, 36). Compoision of β-catenin/TCF complex transcriptional activators, like **BCL**9L, **PG**1, and **PG**2, mediates its activity and target specificity to elicit fundamental patterning processes, involving reciprocal epithelial–mesenchymal interactions during tumorigenesis and normal development (e.g., nervous system; ref. 40). The inverse relationship between proliferation and differentiation is well known and intimately involved in PPGL development. Thus, collective disruption of these factors by aberrant methylation may contribute to PPGL metastases by impairing numerous aspects of nervous system development, such as migration, cellular identity, and differentiation.

Interestingly, we observed aberrant methylation of numerous transcriptional regulators in metastatic PPGLs. In fact, **RDBP** hypermethylation in metastatic PPGLs was highly significant (P = 0.003) as shown by pyrosequencing, which indicated its potential diagnostic utility in the clinical setting. The **RDBP** encodes subunit E of the NELF complex, which mediates transcriptional pausing by cooperative binding to elongating RNA polymerase II (41, 42). Transcriptional pausing represents an important regulatory mechanism for many genes, including **JUNB** (43). Recognized as a key player in neuronal apoptosis as a C-JUN antagonist, depletion of **RDBP** by RNA interference was described to enhance **JUNB** expression in human hepatoma cells (34, 43). Data from T47D cells upon **RDBP** silencing helped us identify potential **RDBP** targets (**JUNB**, **TIMP1**, and **CDKN2C**). Besides its involvement in neuronal apoptosis, **JUNB** has also been reported to increase the invasive and angiogenic potential in numerous tumors, such as renal cell carcinoma, by inducing **MMP**-2/9 (44). **RDBP** knockout decreased the expression of **TIMP1**, which was shown to suppress invasion and metastasis in various human tumors through inhibition of **MMP**-2/9 (45). Another **RDBP** target gene identified in the T47D data, **CDKN2C** has been described as a tumor suppressor involved in PPGL and medullary thyroid carcinoma (46).

Although it was not possible to integrate the gene expression and DNA methylation data due to the low numbers of metastatic PPGLs common in both datasets, we were able to observe downregulation of three genes (**PDZK1**, **RE**G, and **GPX3**) both in the T47D data after **RDBP** knockout and metastatic PPGL. This suggests that expression of these three genes may also represent potential markers for metastatic PPGLs. **GPX3** silencing has been reported in metastatic tumors, such as in gastric carcinomas, where it increased migration and impaired mechanisms regulating reactive oxygen species (47). **RE**G was reported as a prognostic marker in breast cancer, whose expression correlated inversely proliferation, patient survival, and development of distant metastases (48). It has been reported...
that PDZK1 forms a complex linking somatostatin receptors and phospholipase C-β (PLC-β), necessary for somatostatin physiologic responses (49). Although further studies are warranted, epigenetic silencing of RDBP in metastatic PPGL may cause global changes in chromatin and/or gene transcription, possibly affecting expression of genes (e.g., *RUNB, PDZK1, RERG, GPX3*) involved with response to apoptotic stimuli and invasion, proliferation, and metabolism.

In conclusion, this is the first high-throughput study to explore DNA methylation in metastatic PPGL, as well as PPGLs with diverse genetic backgrounds. We demonstrate that DNA methylation patterns differ according to PPGL genotype, and verify SDHx-related tumors display high-CIMP. Also, our results indicate that metastatic PPGLs are not necessarily associated with the high-CIMP previously described in relation to *SDHx/FH* mutations (2, 8). Most importantly, we identified and validated 52 CpGs associated with the development of metastases in two large independent cohorts of these rare tumors. Aberrant methylation of these CpGs could make metastatic PPGLs less sensitive to proapoptotic stimuli and warrants further investigation. Of these, 48 CpGs showed significant associations with TTP. Our bioinformatics analyses and previous experimental evidences suggest that aberrant DNA methylation metastatic PPGLs could affect nervous system development and transcriptional regulation. Finally, *RDBP* hypermethylation was further confirmed in metastatic PPGL by pyrosequencing in an independent series and should be assessed as a new prognostic marker for metastatic PPGL.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


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