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Circulating Tumor DNA (ctDNA) Methylation Quantification as Diagnostic Biomarker of Pheochromocytomas (PCCs) and Paragangliomas (PGLs)

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Abstract: Introduction: Circulating tumor DNA (ctDNA) is newly diagnosed tumor DNA that can easily represent a tumor's genetic and epigenetic change. Pheochromocytomas (PCCs) and Paragangliomas (PGLs) are rare tumors of adrenal gland tissue that have the potential to be detected by ctDNA. We aimed to study the potential of the methylation status of *RDBP*, *SDHB*, and *SDHC* genes in ctDNA of PCCs/PGLs patients as a diagnostic biomarker. **Materials and Methods:** Clinical data, fresh frozen tissue, the blood of 12 PCCs/PGLs patients, and blood of 12 age/sex-matched normal patients were collected. The methylation status of *RDBP*, *SDHB*, and *SDHC* was compared between cases and controls by MS-HRM analysis. **Results:** Amongst six promoter regions of *RDBP*, *SDHB*, and *SDHC,* promoter methylation quantification of *SDHCa* and *RDBPb* was significantly different between PCCs/PGLs and controls. *SDHCa* was methylated in 49.93% of PCCs/PGLs cases vs. 8.33 % of control samples, p-value: 0.026, area under curve AUC=0.757, and *RDBPb* in 74.9% of PCCs/PGLs cases vs. 25.0% of control samples, p-value: 0.032, AUC=0.750. **Conclusions:** This study suggests the ctDNA potential for a less invasive source of tumor epigenetic modification in PCCs/PGLs malignancies. The *SDHCa* and *RDBPb* hypermethylation warrant further exploration as diagnostic tools for PCCs/PGLs.

Keywords: ctDNA; liquid biopsy; Methylation; *SDHCa*; *RDBPb;* Pheochromocytoma; Paraganglioma

Introduction

Pheochromocytomas arise from chromaffin cells in the adrenal medulla, and PGLs arise from chromaffin cells in the ganglia of the autonomic nervous system. Paragangliomas originate from sympathetic or parasympathetic ganglia in the abdomen, thorax, and pelvis (1, 2). The annual incidence of PCCs/PGLs is about 0.8 per 100,000 people (3). Nevertheless, this is probably an underestimation because half of PCCs/PGLs might be diagnosed at autopsy (4). PCCs/PGLs mostly happen in the 3rd and 4th decades of age, equally in both genders (1). There is an extreme need to optimize biomarkers, mainly genetically and epigenetically, to discriminate the PCCs/PGLs and plan the exact treatment strategy at the precise time.

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Recently, the new aspect of Liquid Biopsy (LB) has brought an exceptional tumor marker as the real-time representation of the tumor. Numerous studies focus on genetic and epigenetic circulating tumor DNA (ctDNA) as the tumor markers (5). However, the latest discoveries related to ctDNA genetic modification and circulating tumor cells (CTCs) in PCCs/PGLs are limited (6, 7).

More than genetic modifications, there are some epigenetic variations in DNA molecules that can change the gene expression of the tumoral cells with no change in the exact DNA sequences (8). Intergenic CpG sites are classically methylated in normal tissue. At the same time, they are often hypomethylated in the tumor, resulting in transcriptional changes by interfering with transcription factor binding and changing the euchromatin to heterochromatin (9, 10). DNA methylation patterns in some specific genes are reported to be associated with progression-free survival (PFS) and overall survival (OS) in several cancers (11). Three target genes were selected in this study, RDBP (Negative Elongation Factor Complex Member E) as the essential component of the NELF complex, a complex that negatively regulates the elongation of transcription by RNA polymerase II. SDHB and SDHC encoding subunits of succinate dehydrogenase are significant cause of hereditary paraganglioma and pheochromocytoma and demonstrate that these genes are classic tumor suppressors. Succinate dehydrogenase is a heterotetrameric protein complex and a component of the Krebs cycle and the mitochondrial respiratory chain (succinate: ubiquinone oxidoreductase or complex II). The ctDNAs can be considered a potential non-invasive source of methylation change in PCCs/PGLs. In this study, we examined the methylation quantification of PCCs/PGLs in ctDNA as the tumor transformation biomarker.

Materials and Methods

We ran this study on 12 consecutive PCCs/PGLs patients (cases) and 12 non-cancerous patients (controls). All patients signed the informed consent and all procedures were under National Institute for Medical Science Development Ethics Committee *(IR.NIMAD.REC.1397.452)*. The blood samples were gathered in EDTA-containing vials before surgery for ctDNA analysis. Tumor tissues were captured during surgery in the Department of Surgery in Shariati hospital as the origin of the genomic DNA (gDNA) source. All surgically resected tissues were snap-frozen in liquid nitrogen for further analysis.

Tissue and ctDNA extraction:

Fresh frozen tissues of PCCs/PGLs were removed and kept in liquid nitrogen for a maximum of 1 month, then tissue DNA (gDNA) was extracted using DNeasy Blood & Tissue Kit (Qiagen, Netherlands, Cat No: 69504) (12). For ctDNA extraction, about 4-6 ml blood samples were collected from both PCCs/PGLs and control groups, and the ctDNA extraction was done within two hours. For ctDNA extraction, the plasma was first detached by centrifugation of blood at 2800 rpm for fifteen minutes using the Ficoll separation method. Then, plasma was moved to a sterilized tube, and ctDNA was extracted according to the Norgen Plasma/Serum Cell-Free Circulating DNA Purification Midi Kit protocol (Canada, *Cat No: 55600*). Lastly, the purity and concentration of ctDNA were determined by optical density at 260 and 280nm by Thermo Scientific™ NanoDrop™ spectrophotometers 2000c (Thermo Fisher Scientific Inc.). Both extracted ctDNA and gDNA were stored in the freezer at -80°C for further analysis.

Bisulfite modification

For methylation quantification analysis, ctDNA and its counterpart gDNA from each candidate PCCs/PGLs and ctDNA from controls were treated by the "EZ DNA Methylation-Gold™ Kit" (Zymoresearch, USA, Cat No: D5005) according to the manufacturer's protocol. For the high methylation resolution melting analysis (HRM), six promoter regions of three target genes, *RDBP*, *SDHB*, and *SDHC*, were selected by specific primers (Table 1).

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Table 1. Specific primer sequences for methylation quantification through MS-HRM analysis of six selected promoter regions of *RDBP*, *SDHB*, and *SDHC*.

Gene	Part	Forward Primer	Reverse Primer	T_{ann}	Number of CpG sites in the amplicon
RDBP	a	5' GGTAAGTTTTTTGTTTTTTAT 3'	5' TTTAAATACATATAATTCA 3'	56°C	15
	b	5' GGATATAGTTTGGTTTAAG 3'	5′ ACATCTTTCTCCACTATTAC 3′	52°C	9
SDHB	a	5' GTTAGTGTTTTAGTGGATGT 3'	5' AAACTCACCTACAAACAAAC 3'	57°C	17
	b	5' GGGAAGTTAAATGGGT 3'	5' TCCACTAAAACCCACT 3'	55°C	14
SDHC	a	5' GTAATTAGTTAGGTAGAG 3'	5' ACTAAATCACCTCAACA 3'	50°C	14
	b	5'TAGATGTAGATTTTGAGTTA 3'	5'ACTCTACTAACTAATTTAC 3'	49°C	6

The MS-HRM program includes three main steps: holding step (94°C for 20 minutes), going to 40 cycles of 94°C for 10 seconds, several annealing temperatures depending on the primer (varying from 45°C to 60°C) for 35 seconds, and extension time of 72°C for 35 seconds leading to the final step of the melting curve. The melting curve step was made by heating the samples to 90°C for 15 seconds and 60°C for one minute leading to 65°C for 15 seconds, and afterward continuously heated up to 95°C with the acquisition of data during every 0.3°C rises in temperature.

All reaction mixture consisted of 10µl of master mix (Amplicon, Cat No: A325406), 20 *pmol* of each primer, and 2*µl* (almost 10*ng*) of bisulfite modified DNA template in the whole volume of 20 *µl*. Moreover 0%, 25%, 50%, 75%, and 100% methylated controls were run in each reaction. Standard curves with identified methylation ratios were comprised in all assays by assuming the methylation ratio of the unknown target. The ABI Step One Plus system did MS-HRM tests in triplicates.

Statistical analysis

Samples were considered hyper-methylated for curves between 25% and 100% of methylation compared to controls and non-methylated when the methylation amount was near 0% compared to the control. The comparison was made between two main groups: the case group comprising 12 PCCs/PGLs patients and the control group comprising 12 non-cancerous patients. The specificity and sensitivity of each target CpG site were assessed through a 2×2 table. The correlation of mutation and methylation of ctDNAs and their tissue counterparts were performed through spearman correlation analysis, and ctDNA's methylation of PCCs/PGLs patient's Receiver Operating Characteristics (ROC) curves were constructed. Statistical Package did all statical analyses for Science Software (SPSS) (*version 17.0; SPSS Inc. Chicago, Illinois*).

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Results

Demographic characteristics of PCCs/PGLs patients and controls are presented in Table 2. The age of all patients ranged from 26 to 60. Among all 12 PCCs/PGLs, tumor size varied from 0.5-6 cm, and three patients (25%) were defined as homogeneous and hypoechoic solid lesions with well-defined borders. PCCs/PGLs patients showed significantly higher systolic blood pressure (SBP), diastolic blood pressure (DBP), and also considerably lower weight and body mass index (BMI).

Table 2. Demographics of PCCs/PGLs (cases) and non-cancer patients (controls).

Variables	PCCs/PGLs cases (n= 12)	Controls (n=12)	P-value
Age (years)	41.25 (±10.532)	42.42 (±11.828)	-
Gender: Female	8 (66.7%)	8 (66.7%)	-
Male	4 (33.3%)	4 (33.3%)	
Weight (kg)	61.58 (±5.299)	74.00 (±11.201)	0. 002
Height (cm)	165.50 (±9.200)	165.32 (±9.55)	0.370
BMI	23.68 (±3.884)	27.05 (±3.833)	0.043
SBP (mm/Hg)	13.72(±1.190)	11.50 (±1.167)	<.001
DBP (mm/Hg)	10.36(±1.68)	7.91 (±1.37)	0.001
Educated (After High School)	7 (58.3%)	10 (83.3%)	0.146
Malignant	5(41.6%)	-	-
Tumor size	0.8-12 cm	-	-
Norepinephrine level	4.2 ± 1.5%	-	-
Epinephrine	58.02(±5.12)	-	-
Norepinephrine	61.3 (±3.459)	-	-
Dopamine	220 (±2.023)	-	-

BMI: body mass index, **SBP**: systolic blood pressure**, DBP**: diastolic blood pressure.

For each run of MS-HRM, ten wells were allocated to the methylation controls 0%, 25%, 50%, 75%, and 100%, and test samples were run in triplicate. The melting curve was considered for additional analysis to determine the methylation status (Figure 1). The methylation level of each sample was estimated by comparing each sample's normalized melt curve with the controls' normalized melt curves.

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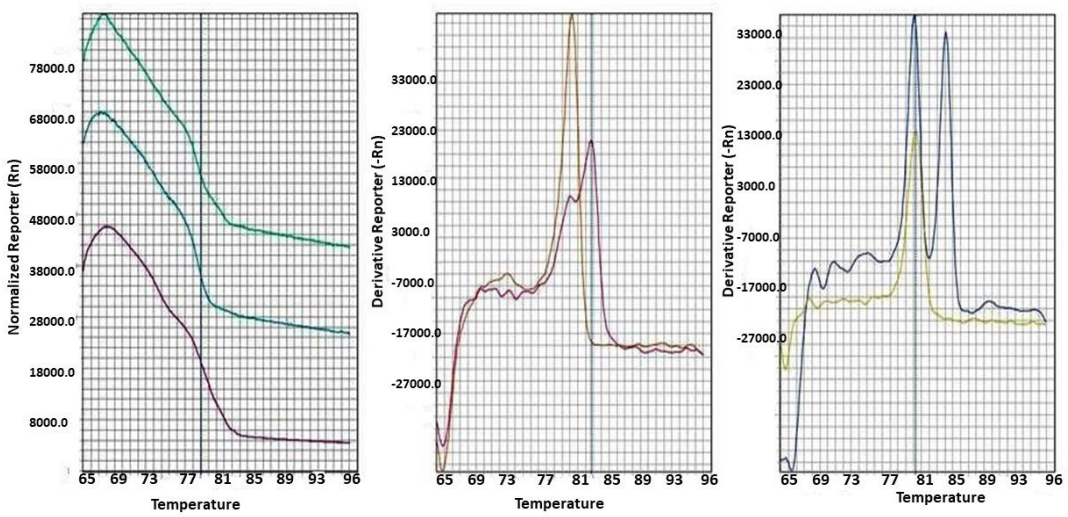

Figure 1. The MS-HRM for studying the methylation status of six CpG sites of three target genes.

The methylation status of all six target regions in the case and controls are presented in Table 3 and Figure 2. Among six promoter regions within three candidate genes, the *SDHCa* (the promoter region harboring initial ATG code) and *RDBPb* methylation statuses were hypermethylated (more than 25 % methylation) in PCCs/PGLs patients compared to the control group, and p-value 0.026 and 0.03, respectively (13). Bisulfite Pyrosequencing confirmed all results on tumor tissue DNA as an additional technique.

Table 3. Methylation pattern of six target promoter regions in PCCs/PGLs and normal cases.

Promoter Region	Methylation	PCCs/PGLs patients Number (percentage)	Control (percentage)	P-value
SDHBa	0-12.5% methylated (non-methylated)	8 (66.6%)	7 (58.3%)	0.886
	12.5 ≤, <25% methylated	1 (8.33%)	3 (25.0%)	
	25≤, <50% methylated	1 (8.33%)	0 (0.0%)	
	50≤, <75 % methylated	1 (8.33%)	1 (8.33%)	
	75- 100% methylated	1 (8.33%)	1 (8.33%)	
SDHBb	0-12.5% methylated (non-methylated)	3 (25.0%)	3 (25.0%)	0.507
	12.5 <, ≥25% methylated	1 (8.33%)	2 (16.6%)	
	25≤, <50% methylated	2 (16.6%)	5 (41.6%)	
	50≤, <75 % methylated	3 (25.0%)	1 (8.33%)	
	75- 100% methylated	3 (25.0%)	1 (8.33%)	
SDHCa	0-12.5% methylated (non-methylated)	4 (33.3%)	9 (75.0%)	0.026*
	12.5 ≤, <25% methylated	2 (16.6%)	2 (16.6%)	
	25≤, <50% methylated	2 (16.6%)	1 (8.33%)	
	50≤, <75 % methylated	1 (8.33%)	0 (0.0%)	
	75- 100% methylated	3 (25.0%)	0 (0.0%)	

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	0-12.5% methylated (non-methylated)	7 (58.3%)	5 (41.6%)	0.750
	12.5 ≤, <25% methylated	0 (0.0%)	2 (16.6%)	
SDHCb	25≤, <50% methylated	1 (8.33%)	2 (16.6%)	
	50≤, <75 % methylated	0 (0.0%)	1 (8.33%)	
	75- 100% methylated	4 (33.3%)	2 (16.6%)	
	0-12.5% methylated (non-methylated)	11 (891.6%)	10 (83.3%)	0.987
	12.5 ≤, <25% methylated	1 (8.33%)	1 (8.33%)	
RDBPa	25≤, <50% methylated	0 (0.0%)	1 (8.33%)	
	50≤, <75 % methylated	0 (0.0%)	0 (0.0%)	
	75- 100% methylated	0 (0.0%)	0 (0.0%)	
	0-12.5% methylated (non-methylated)	3 (25.0%)	7 (58.3%)	
	12.5 ≤, <25% methylated	0 (0.0%)	2 (16.6%)	
RDBPb	25≤, <50% methylated	2 (16.6%)	0 (0.0%)	0.032*
	50≤, <75 % methylated	3 (25.0%)	3 (25.0%)	
	75- 100% methylated	4 (33.3%)	0 (0.0%)	

*If the p-value is less than 0.05, we reject the null hypothesis that there's no difference between the two groups and conclude that a significant difference does exist. *If at least one targeted promoter region was methylated more than 25%, the final methylation status of the target gene was hypermethylated.

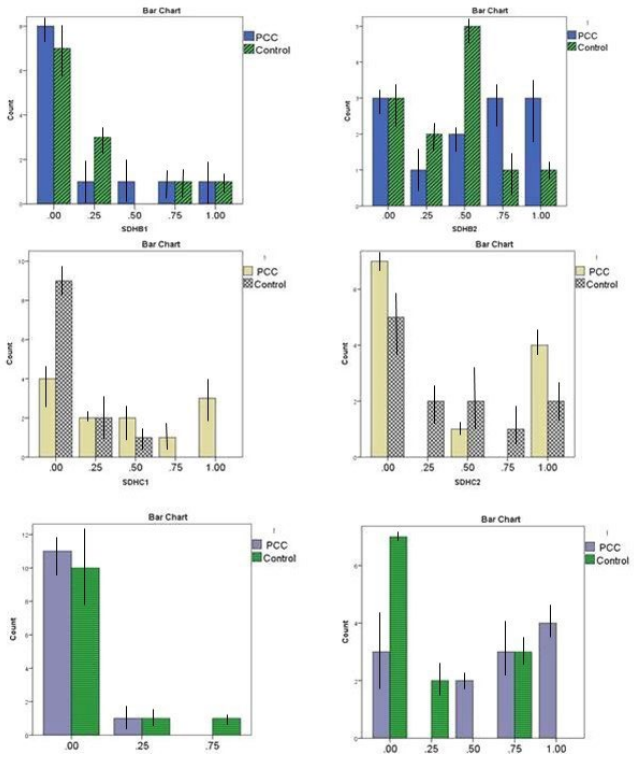

Figure 2. Comparison of methylation quantification in six target *promoter regions* of *SDHB*, *SDHC*, and *RDBP* genes between PCCs/PGLs cases and non-cancerous patients. PCC is an abbreviation of PCCs/PGLs.

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The maximum value for kappa observed was 0.919 between *SDHBb* and *SDHCa* and 0.805 between *RDBPb* and *SDHCb* (Table 4).

Table 4. Observed agreements between six CpG sites of *SDHB*, *SDHC*, and *RDBP2* in plasma ctDNA.

	RDBP1	***RDBP2***	***SDHB1***	***SDHB2***
SDHCa	.567	.668	.715	.919 *
SDHCb	.624	.824 *	.805	.649

According to possible interpretation of Kappa, Poor agreement = Less than 0.20, Fair agreement = 0.20 to 0.40, Moderate agreement = 0.40 to 0.60, Good agreement = 0.60 to 0.80 and, Very good agreement = 0.80 to 1.00. *The highest kappa agreement score.

ROC curves were plotted based on the percentage of methylated reference (PMR) values in PCCs/PGLs and the control group to indicate if any of the targeted six promoter regions in circulating plasma ctDNA can be considered as PCCs/PGLs specific diagnostic markers (Figure 3).

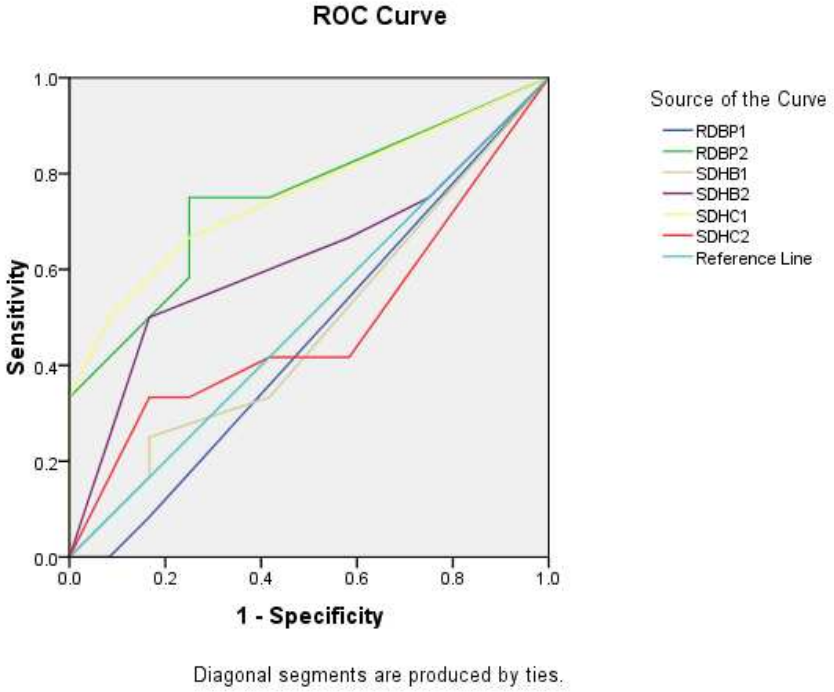

Figure 3. Receiver Operating Characteristic (ROC) curves for the DNA methylation markers (as ranked by p-value), using the current collection of circulating plasma ctDNA.

The sensitivity and specificity of target CpG sites were defined based on correctly classifying subjects with methylated promoters into PCCs/PGLs. The area under the curve (AUC) ranged from 0.455 in *RDBPa* to 0.757 in *SDHCa* and then 0.750 in *RDBPb* (Table 5).

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Table 5. AUC, Sensitivity & Specificity Analysis for six targeted promoter regions of PCCs/PGLs from patients' *ctDNA.*

The ctDNA promoter Locus	AUC (95 % CI)	Sensitivity	Specificity
SDHBa	0.476 (0.240-0.712)	25%	83.3%
SDHBb	0.622 (0.388 - 0.855)	66.6%	41.6%
SDHCa	0.757 (0.558 - 0.956)	50%	91.6%
SDHCb	0.483 (0.242–0.723)	41.6%	58.3%
RDBPa	0.455 (0.220 – 0.690)	21%	91.6%
RDBPb	0.750 (0.549 - 0.951)	75%	75%

Discussion

Several genetic mutations are considered important molecular diagnostic biomarkers in PCCs/PGLs. Our pioneering study represents the role of specific genes DNA methylation as a PCCs/PGLs potential diagnostic tool. Herein, we report *SDHC1* hypermethylation in 49.93% of PCCs/PGLs cases vs. 8.33 % of control samples, p-value: 0.026; and *RDBP2* promoter region 74.9% hypermethylation in cases vs. 25.0% in controls, p-value: 0.03. These target regions have AUC 0.750 in *RDBP2* and 0.757 in *SDHC1* promoters.

Changes in gene expression patterns that trigger the cell to malignancy can result from epigenetic modification (14, 15). Methylation-Sensitive High Resolution Melting (MS-HRM) is an in-tube; PCR-based method to detect methylation levels at specific loci of interest. A unique primer design facilitates a high sensitivity of the assays enabling detection of down to 0.1-1% methylated alleles in an un-methylated background. The only study so far in which the ctDNA was challenged as a PCCs/PGLs tumor genetic indicator was done by Wang et al. in 2018, and to the best of our knowledge, our study is the only one that seeks methylation of ctDNA (6). Nowadays, it has become common knowledge that ctDNA, as the liquid biopsy main component, has excellent potential to non-invasively show the tumor's genetic status (16-19).

The global DNA methylation array indicates three distinct clusters: M1–3. M1 comprises tumors with *SDHx* mutations and hypermethylation, M2 *VHL*-mutated tumors, and M3 tumors with *NF1* and *RET* mutations and hypomethylation (11, 20). Epithelial to Mesenchymal Transition *(EMT)* can be activated in metastatic PCCs/PGLs by *SDHB* gene mutations (21). It was shown by Astuti et al. that *SDHB* was hypermethylated in 21% of primary neuroblastomas and 32% of PCCs/PGLs (22). Our results indicate 25.0% *SDHB* promoter region hypermethylation and more than 75% of CpG sites harboring initial ATG.

However, based on posterior microsatellite instability and hypermethylation promoter studies, there is still doubt that *SDHB* methylation can play a role once it is unlikely to be related to either tumor initiation or progression in neuroblastoma (23). We have considered two upstream promoter regions of initial ATG with no difference in methylations between PCCs/PGLs and control patients. Contradictory, it was reported that *EMT* hypermethylation and *in vitro* acquisition of metastatic properties could be the consequence of *SDHB* loss of function (24). *SDHB* mutation might change the methylation status of methyltransferase enzymes like MGMT (25-28).

Considering gene silencing by promoter CpG methylation as an essential epigenetic mechanism in tumorigenesis, our results indicate that *SDHC* promoter hypermethylation could play a role in developing PCCs/PGLs. The altered expression of SDHC has been reported several times as the consequence of genetic and epigenetic changes (29-32). *SDHC* promoter methylation can cause *SDHC* inactivation, so it is essential to consider epigenetic changes and functional readouts in the genetic evaluation of patients (33). *SDH*-loss cells are selectively vulnerable to *LDH* genetic knock-down or chemical inhibition, suggesting that *LDH* inhibition may be an effective therapeutic strategy

for *SDH*-loss and *SDHC*-loss transcriptional change correlate with baseline expression values in normal cells (34). Our results indicate that the CpG sites far from initial ATG are hypermethylated in PCCs/PGLs, and its methylation agrees with *SDHB* and *RDBP*. The *RDBP* gene is responsible for coding negative elongation factor E (NELF) as a complex that negatively regulates the elongation of transcription by RNA polymerase II (35). The first large-scale study of DNA methylation in metastatic PGL by de Cubas and colleagues supports that *RDBP* could be used for stratifying patients according to the risk of developing metastases (36).

Our study indicated *RDBP* promoter hypermethylation in PCCs/PGLs, in line with Backman et al., which suggested hypermethylated *RDBP* in metastasizing PGLs regardless of mutational status (11). *RDBP* hypermethylation should be further explored as malignancy and survival markers in patients with PCCs/PGLs (37-39). *RDBP* methylation can trigger the PCCs/PGLs to be malignant, and interestingly in 5 malignant patients in our study, the CpG sites in promoter regions harboring ATG were hypermethylated, similarly to Yong Joon Suh et al. findings (40). *RDBP* methylation status can predict outcome in PCCs/PGLs and the potential of targeted therapy (37, 41). Unlike *SDHx* which are methylated or mutated in several tumor types, the *RDBP* hypermethylation has been just reported in PCCs/PGLs and of the initial 86 candidate CpGs, from 47 genes, just *RDBP* was confirmed and could be used for stratifying patients according to the risk of developing metastases (36). However, this type of test still has significant limitations since it is only possible to be performed in centers with the technology available implicating in higher hospital structure and investment, which is not (yet) the reality of many centers worldwide. The primary study limitation was that a genetic analysis of ctDNA is very challenging because ctDNA is often highly fragmented and often limited in yield.

Conclusions

In line with current literature, our results support *SDHCa* and *RDBPb* hypermethylation in ctDNA of PCCs/PGLs as potential diagnostic and prognostic tools.

Author Contributions: S.M.K.A. and R.H. are principal investigators, L.O.R. edited the manuscript, M.A.P. and M.E. analyzed the data, S.H.N. and S.M.T. provided data and data curation, and F.K.H. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest: All authors declare there is not any conflict of interest for this publication.

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