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PCDHGC3 promoter methylation identifies malignancy and poor prognosis in adrenocortical tumors

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Abstract

Background

Reliable biomarkers to distinguish adrenocortical carcinoma (ACC) from benign adrenal adenoma (AD) and to stratify postoperative risk remain an unmet clinical need. *PCDHGC3*, a clustered protocadherin previously linked to aggressiveness in endocrine malignancies, has not been investigated in ACC.

Results

Here, we analyzed *PCDHGC3* promoter methylation in 60 surgically resected adrenal tumors (48 ACCs and 12 ADs) to assess its diagnostic and prognostic value. Tumors were classified according to *PCDHGC3* promoter methylation status using an optimized threshold. Hypermethylation was detected in 87.5% of ACCs and in none of the ADs ($p < 0.0001$) achieving 100% specificity and 87.5% specificity. Methylation status was independent of age, sex, tumour size, ENSAT stage, Ki-67 index and Weiss score. Among ENSAT I–III ACCs, *PCDHGC3* promoter methylation predicted shorter event-free survival (median 11.4 vs 31.0 months, $p = 0.027$), while Ki-67 > 20% was associated with worse overall survival. These findings were externally validated in the TCGA ACC cohort, where *PCDHGC3* hypermethylation and low mRNA expression similarly correlated with poor prognosis. In SW13 ACC cells, *PCDHGC3* was found epigenetically silenced through promoter methylation and decitabine treatment restored its expression. Functional assays revealed that *PCDHGC3* re-expression induced G1-phase arrest supporting a tumor suppressor role.

Conclusions

PCDHGC3 promoter methylation is a highly specific biomarker of malignancy in adrenocortical tumours and identifies a biologically aggressive ACC subset, supporting its potential clinical utility as a rule-in diagnostic and risk-stratification tool.

Background

Adrenocortical carcinoma (ACC) is a rare but highly aggressive malignancy arising from the adrenal cortex, with an annual incidence of approximately 0.7–2 cases per million individuals [1]. Prognosis remains poor, with five-year survival rates below 50% in most cases [2]. A major clinical challenge is distinguishing ACC from benign adrenocortical adenomas (ADs), particularly in the context of adrenal incidentalomas [1, 3]. Although most adrenal masses are benign, up to 10% of incidentalomas may represent ACCs, and clinical, radiologic, and histopathologic features often overlap, rendering accurate diagnosis challenging in borderline cases [1].

The Weiss scoring system and the Ki-67 proliferation index are routinely used to differentiate malignant from benign adrenal tumors, with a Weiss score ≥ 3 being indicative of ACC [2, 4]. In addition, the reticulin algorithm and the Helsinki score have been introduced to improve diagnostic accuracy, particularly in borderline cases, by integrating architectural disruption, mitotic activity, necrosis, and proliferative indices [5–7]. Despite these advances, interobserver variability, limited sensitivity in low-grade tumors, and suboptimal performance in certain histological variants remain significant challenges [8–11]. These limitations underscore the urgent need for objective, reproducible molecular biomarkers that can complement histopathological evaluation and improve both diagnostic and prognostic stratification of adrenocortical neoplasms.

Epigenetic alterations, particularly aberrant DNA methylation, have emerged as promising tools for cancer classification and risk stratification [12, 13]. In ACC, genome-wide methylation studies have identified distinct DNA hypermethylation patterns that not only discriminate ACCs from ADs but also define a CpG island methylator phenotype (CIMP) associated with poor prognosis [14–20].

Among the epigenetically regulated gene families in cancer, increasing attention has focused on the protocadherin (PCDH) gene clusters located on chromosome 5q31. These genes encode cell adhesion molecules frequently silenced by promoter hypermethylation in a variety of malignancies [21–23]. Within this family, *PCDHGC3* has emerged as a potential tumor suppressor, particularly in neuroendocrine neoplasms. Recent evidene links *PCDHGC3* promoter hypermethylation to aggressive tumor features, including a strong association with metastatic behavior in pheochromocytomas and paragangliomas (PPGLs) [24], and higher histological grade in gastrointestinal neuroendocrine tumors [25]. Notably, Bernardo-Castiñeira *et al.* [24] reported frequent *PCDHGC3* hypermethylation in metastatic PPGLs, while Cubiella *et al.* [25] found that its hypermethylation is significantly enriched in high-grade gastrointestinal neuroendocrine carcinomas and independently associated with poor overall survival, reinforcing its potential as a prognostic biomarker. Beyond neuroendocrine tumors, recent work has identified *PCDHGC3* as a key regulator of tumor progression in clear cell renal cell carcinoma, where its loss promotes proliferation, metastatic dissemination, and metabolic reprogramming through activation of FAK and mTOR–HIF2α signaling pathways [26]. Together, these findings support a broader role for *PCDHGC3* as a context-dependent tumor suppressor, whose epigenetic silencing may represent a common mechanism underlying tumor aggressiveness across distinct malignancies.

Here, we sought to determine whether *PCDHGC3* methylation serves as a reliable biomarker of malignancy in ACC. We further evaluated its association with clinicopathological features and assessed its diagnostic and prognostic utility. In addition, we performed functional assays in ACC cells to explore the biological consequences of *PCDHGC3* silencing and its potential reversibility through epigenetic modulation.

Methods

Patient cohort, tumor samples, and clinical data

This retrospective study included 60 adrenocortical tumor samples collected between 2003–2022 from five hospitals and biobanks in Spain. Participating biobanks comprised the Principado de Asturias BioBank (BioPA) (B.0000827, Spanish National Biobanks and Biomodels Network, PT20/0161), affiliated with the Hospital Universitario Central de Asturias (HUCA, Oviedo); the HCB-IDIBAPS Biobank (B.0000575, Platform ISCIII Biobanks and Biomodels) affiliated with the Hospital Clinic de Barcelona and the August Pi i Sunyer Biomedical Research Institute (IDIBAPS), and the Hospital Ramón y Cajal-IRYCIS Biobank (B.0000678, ISCIII Biobanks and Biomodels Platform, PT23/00098) affiliated with the Hospital Universitario Ramón y Cajal and the Ramón y Cajal Institute for Health Research (IRRYCIS, Madrid). Formalin-fixed paraffin-embedded (FFPE) or fresh-frozen tumor tissues were included based on sample availability and DNA quality. Clinical and pathological data, including age, sex, tumor size, Weiss score, ENSAT stage, and Ki-67 index, were retrieved from medical records and pathology reports. Tumors were classified according to the ENSAT staging system and Weiss criteria. All samples were processed according to standardized operating procedures with approval from the corresponding Institutional Ethical and Scientific Committees. All methods were performed in accordance with relevant guidelines and regulations, and with the principles of the Declaration of Helsinki (1975), as revised in 1983.

Given the rarity and histological heterogeneity of ACC, the cohort was deliberately enriched for ACC cases (n = 48) while retaining a reference set of well-characterised ADs (n = 12). ADs were confirmed by stringent histopathological criteria to ensure an unequivocally benign comparator. This asymmetric design maximised power for biomarker performance across the spectrum of ACC without compromising diagnostic specificity.

Cell culture, transfection, and functional assays

SW13 ACC cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 2 mM L-glutamine, and 100 µM non-essential amino acids, and maintained at 37°C in a humidified incubator with 5% CO_2. For DNA demethylation assays, cells were treated with 5 µM decitabine (Sigma-Aldrich) for 1 to 6 days, with medium and drug refreshed daily. The cell line was authenticated by short tandem repeat profiling and routinely tested for human pathogens and mycoplasma.

For *PCDHGC3* overexpression, cells were transiently transfected with a mouse-derived PCDHGC3-GFP vector (FL-C3-GFP) [27] using Lipofectamine LTX (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Empty vector was used as control. Transfection efficiency and subcellular localization were confirmed by fluorescence microscopy.

Cell cycle analysis was performed 48 hours post-transfection. Cells were fixed with 70% cold ethanol for 90 minutes, washed twice with PBS, and stained with 1 µg/ml DAPI before flow cytometry. Experiments were performed in biological triplicates unless otherwise stated. Data were analyzed using FlowJo software.

Gene expression analysis by RT- quantitative PCR

Total RNA was extracted using the mirVana™ miRNA Isolation Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was synthesized from 100 ng of RNA using the Maxima First Strand cDNA Synthesis KIT for RT-qPCR" (ThermoFisher). Gene expression was analyzed using TaqMan PCR Master Mix (Applied Biosystems, Waltham, MA, USA). Peptidylprolyl isomerase A (PPIA) mRNA was used to normalize RNA input. Relative quantification was performed in triplicate using the $2^{-\Delta\Delta CT}$ method.

DNA methylation analysis

Genomic DNA was extracted using the QIAamp DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were assessed using a Nanodrop spectrophotometer. Bisulfite conversion of DNA was performed using the Z DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA) which converts unmethylated cytosines to uracils while preserving methylated cytosines. The target region was PCR-amplified using specific primers (5'-GGGATGAGGTAGAGATTGAATAG-3'; and 5'-CCTCCAAACCTCTAAAACCATCTCA-3'). Pyrosequencing was carried out using the PyroMark Q24 Advanced System® (QIAGEN) with a specific primer (5'-GAGGTAGAGATTGAATAGT-3'). Primers were designed using the PyroMark Assay Designer (QIAGEN), focusing on the promoter region of the *PCDHGC3* gene (TSS200 = 0–200 bases upstream of the transcription start site). The average methylation percentage across eight CpG sites was used for analysis.

For external validation, *PCDHGC3* methylation and mRNA levels were retrieved from the TCGA cohort of ACCs via the UCSC Xena Browser. Patients were dichotomized based on the mean methylation beta value (0.046) and the median mRNA levels (12.38, log2(norm_count + 1)). Survival analyses were performed accordingly.

mRNA expression data for *PCDHGC3* across cancer cell lines were retrieved from the DepMap Portal (https://depmap.org/portal/) [28], using the Expression Public 24Q4 dataset.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation. Categorical variables were analyzed using the Chi-squared test or Fisher's exact test, as appropriate. Methylation and expression levels were compared using unpaired t-tests with Welch's correction or non-parametric tests, based on data distribution and variance. Differences in variance were assessed using the F-test. Receiver operating characteristic (ROC) curve analyses were conducted to determine diagnostic performance and to calculate area under the curve (AUC), sensitivity, specificity, and optimal thresholds based on the Youden index. Bootstrap resampling (1,000 iterations) was applied to internally validate diagnostic metrics. Associations with clinicopathological variables were explored using linear regression and Fisher's exact

tests. Kaplan–Meier survival curves were used to assess prognostic associations, with statistical significance determined by the log-rank test. Event-free survival (EFS) was defined as the time from complete surgical resection of the primary tumor to the first radiological evidence of recurrence. Overall survival (OS) was definedas the time from surgery to death from any cause or last follow-up. Cox proportional hazards regression was performed for variables found significant in univariate analysis. A two-tailed *P*-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism (version 10.5), R (version 4.3.2), and SPSS Statistics (version 29, IBM Corp., Armonk, NY, USA).

Results

Patient cohort and tumor classification

A total of 60 histologically confirmed adrenocortical tumors were analyzed, comprising 48 ACCs and 12 ADs. Clinical and pathological characteristics of the overall sample are summarized in Table 1. ACCs were large (mean diameter 11.3 cm vs. 6.3 cm), showed high proliferative activity (mean Ki-67: 28.5%), and commonly presented at advanced stages (62% ENSAT stage III–IV). Among ACC patients, 41.3% developed recurrence or metastasis after a median follow-up of 61.4 ± 80.75 months, with mean EFS and OS.

Table 1
Clinicopathological characteristics and clinical outcomes of patients with adrenocortical tumors

	ACC	AD
	n = 48	n = 12
Sex (females/males)	32 (66.7%) / 16 (33.3%)	7 (58.3%) / 5 (41.7%)
Age at diagnosis (years)	55.1 ± 14.8 (19–77)	58.4 ± 8.3 (43–76)
Tumor size (cm)	11.3 ± 6.2 (1.7–25.0)	6.3 ± 2.4 (4–10)
Ki-67 (%)	28.5 ± 22.2% (3–80%)	
Histological subtype, n = 39		
-Conventional	31 (79.5%)	
-Oncocytic	4 (10.3%)	
-Sarcomatoid	1 (2.6%)	
-Myxoid	3 (7.7%)	
Weiss index, n = 38		
-<6	16 (42,1%)	
-≥6	22 (57,9%)	
ENSAT stages		
-I	2 (4%)	
-II	15 (31%)	
-III	16 (33%)	
-IV	15 (31%)	
Helsinki score, n = 39		
-<8	3 (10%)	10 (100%)
-≥8	26 (90%)	0 (0%)
Reticulin algorithm, n = 18		
-Negative	8 (50%)	10 (100%)
-Positive	8 (50%)	0 (0%)
Recurrence or metastasis	19 (41.3%)	0 (0%)
EFS (months)	18.3 ± 3.7 (11–25)	

	ACC	AD
	n = 48	n = 12
Number of deaths	27 (56.3%)	
OS (months)	124.3 ± 22.4 (80–168)	

Continuous variables are expressed as mean ± standard deviation (SD); categorical variables are presented as absolute counts and percentages

PCDHGC3 methylation levels discriminate ACC from AD

Analysis of *PCDHGC3* methylation revealed significantly higher mean levels in ACCs (2.81 ± 2.56) compared to ADs (0.92 ± 0.13) ($p < 0.0001$). Methylation variance was also significantly greater in ACCs (F-test, $p < 0.0001$), indicating increased epigenetic heterogeneity in malignant tumors, consistent with partial or cell subset-restricted promoter methylation. As shown in Fig. 1A, while most ADs exhibited low methylation values (< 2%), a subset of ACCs showed markedly elevated levels, with several outliers exceeding 7%.

To establish a threshold for classifying tumors as methylated or un-methylated, we performed receiver operating characteristic (ROC) curve analysis. Using continuous methylation values, the AUC was 0.908 ± 0.039 [95% confidence interval (CI): 0.831–0.985; $p < 0.001$; Fig. 1B].

Applying this predefined threshold, the binary classifier achieved an AUC of 0.938 ± 0.030 (95% CI: 0.878–0.997), with sensitivity of 87.5% (95% CI: 75.3%–94.1%) and a specificity of 100% (95% CI: 75.8%–100%). The positive predictive value (PPV) was 100% (95% CI: 91.6%–100%), and the negative predictive value (NPV) was 66.7% (95% CI: 43.7%–83.7%).

Stratification by histological subtype revealed differences in the prevalence of *PCDHGC3* hypermethylation. Using the same cutoff, hypermethylation was detected in 87% of conventional ACCs, 100% of oncocytic ACCs, and in the single sarcomatoid ACC, whereas none of the myxoid ACCs (0/3) showed elevated methylation levels.

We performed stratified bootstrap resampling (1000 replicates) to estimate 95% CIs for the AUC and diagnostic parameters at the prespecified methylation threshold (Table 2). The optimism-adjusted AUC was 0.91 ± 0.04 (95% CI: 81.8%–97.8%; $p = 0.04$), confirming the robust discriminative performance of *PCDHGC3* methylation. At the fixed cutoff, the bootstrap-derived mean sensitivity was 88% (95% CI: 76%–92%), specificity 100% (95% CI: 75%–100%), PPV 100% (95% CI: 91.8%–100%), and NPV 67% (95% CI: 45.1%–83.8%).

Table 2
Diagnostic performance of *PCDHGC3* promoter methylation for discriminating ACC from AD

Analysis	Cohort description	AUC ± SD (95% CI)	Threshold (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Initial	Full cohort (n = 60)	0.908 ± 0.39 (0.831–0.985)	1.12	87.8	100	100	66.7
Bootstrap (1000 resamplings)	Internal validation	0.913 ± 0.037 (0.835–0.979)	1.12	87.9	100	100	67.1

AUC: area under the curve; PPV: positive predictive value; NPV: negative predictive value. Results from 1000 bootstrap iterations.

Clinical correlations and prognostic value of PCDHGC3 promoter methylation in ACC

We first explored the potential clinical and pathological correlations of *PCDHGC3* methylation levels using multivariate linear regression. The model included age, sex, tumor size, ENSAT stage, Ki-67 index, and Weiss score, but none of these variables showed a statistically significantly association with continuous methylation levels ($R^2 = 0.208$; $p = 0.418$, Table 3). A reduced model including only sex, identified as the strongest (though non-significant) predictor in the full model, also failed to reach significance ($p = 0.114$). These findings suggest that PCDHGC3 promoter methylation is not a linear surrogate of established clinicopathological parameters.

Table 3
Multiple linear regression coefficients for predictors of *PCDHGC3* methylation

Variable	**Coefficient (β)**	**SE**	***t***	***p***
Intercept	-0.059	0.604	-0.10	0.92
Age (per year)	0.009	0.007	1.2	0.24
Sex	0.41	0.247	1.66	0.11
Tumour size (cm)	0	0.039	0	1
ENSAT stage (I–IV)	-0.084	0.111	-0.75	0.46
Ki-67 (% per unit)	0.001	0.003	0.41	0.69
Weiss score	-0.053	0.088	-0.60	0.55
Model	**R^2**	**Global F-test p**		**N**
Full linear (all covariates)	0.208	0.418		28
Reduced model (sex only)	0.069	0.114		34

To explore factors associated with high methylation status, we performed a binary logistic regression using the predefined cutoff value. Consistent with the linear regression analysis, none of the evaluated clinical or pathological variables, including age, sex, tumor size, ENSAT stage, Ki-67 index, and Weiss score, were significantly associated with methylation status (Table 4).

Table 4
Predictors of *PCDHGC3* hypermethylation by binary logistic regression

Variable	**Odds ratio**	**95% CI**	***p***
Intercept	0	0.00–0.62	0.044
Age	1.05	0.97–1.15	0.226
Sex	7.75	0.17–350	0.232
Tumour size	1.05	0.79–1.40	0.716
ENSAT stage	0.55	0.20–1.54	0.256
Ki-67	0.98	0.93–1.03	0.392
Weiss score	0.72	0.36–1.44	0.352
Model	**McFadden pseudo-R^2**	**Likelihood-ratio p**	**N**
Logistic (hyper- vs hypo-)	0.3	0.31	31

These findings suggest that *PCDHGC3* methylation represents an independent molecular event not directly explained by conventional clinicopathological features in this cohort

To evaluate the prognostic relevance, we examined EFS and OS in relation to methylation status. In patients with localized disease (ENSAT stage I–III tumors), those with methylated tumors had significantly shorter EFS (11.4 ± 1.5 months; 95% CI: 8.27–14.5) compared to those with unmethylated tumors (31.0 ± 10.4 months; 95% CI: 10.5–51.4; p = 0.027, Fig. 2).

No significant associations with EFS were found for other clinicopathological variables, including sex (p = 0.336), age ≥ 50 (p = 0.470), Weiss score > 6 (p = 0.709), Ki-67 > 20% (p = 0.182), or ENSAT stage III (p = 0.980). In a Cox regression model adjusted for age and sex, *PCDHGC3* hypermethylation showed a strong trend toward shorter EFS but did not reach statistical significance (HR: 8.082; 95% CI: 0.949–68.818; p = 0.056).

Regarding OS, no significant difference was observed between methylated and unmethylated tumors (147.8 ± 28.1 months; 95% CI: 92.7–202.8 vs 157.4 ± 61.5 months; 95% CI: 36.7–278.1; P = 0.916). However, Ki-67 ≤ 20% was associated with significantly longer survival compared to Ki-67 > 20% (121.5 ± 22.3 vs. 31.86 ± 7.3 months; p = 0.011). No other variable showed significant association with OS, including sex (p = 0.683), age ≤ 50 years vs. > 50 years (p = 0.061), Weiss score > 6 vs. ≤6 (p = 0.490) or ENSAT stage (p = 0.240). In Cox regression Ki-67 index > 20% remained an independent predictor of poor survival (p = 0.042) whereas age ≥ 50 years was not significant (p = 0.166)

External validation using the TCGA cohort

To assess the external validity of our findings, we analyzed *PCDHGC3* promoter methylation in ACC samples from the TCGA dataset. Using the mean methylation value across tumors (0.046) as a threshold, samples were stratified into high and low methylation groups. The high methylation group exhibited mean methylation levels of 0.5016 ± 0.2254, with a median of 0.5916 (95% CI of 0.2650–0.7381). In contrast, the low methylation group showed minimal methylation (0.01004 ± 0.0024), with a narrow range and low variability (coefficient of variation: 23.9%, Fig. 3A).

These data confirm the presence of a subset of TCGA ACCs with significantly elevated *PCDHGC3* methylation, consistent with the pattern observed in our primary cohort. Notably, the methylation levels in the TCGA high group closely mirrored those of methylated ACCs in our study, both in magnitude and in distribution. This supports the biological relevance and reproducibility of *PCDHGC3* methylation as a potential molecular feature of a specific ACC subset.

Next, we explored the prognostic relevance of *PCDHGC3* status. Although high methylation levels showed a trend toward shorter OS, this association did not reach statistical significance (p = 0.066) (Fig. 3B). In contrast, low *PCDHGC3* mRNA expression was significantly associated with worse patient prognosis (p = 0.0047, Fig. 3C), reinforcing the potential role of this gene in ACC progression and outcome.

Importantly, reanalysis of RNA-seq data from the ACC-TCGA cohort revealed that *PCDHGC3* expression was significantly reduced in tumors classified as CIMP-high, a molecular subgroup previously associated with aggressive clinical behavior and poor outcome. Specifically, *PCDHGC3* was downregulated in CIMP-high ACCs compared with non–CIMP-high tumors (log2 fold change = − 0.8, adjusted p = 0.014), linking *PCDHGC3* silencing to the global CpG island hypermethylation phenotype described in ACC [20].

PCDHGC3 silencing and functional relevance in SW13 cells

To further support our findings in tumor tissues, we examined *PCDHGC3* expression across cancer cell lines using data from the DepMap Portal (https://depmap.org/portal/) [28]. Notably, the ACC cell line SW13 ranked among those with the lowest *PCDHGC3* mRNA expression levels (Fig. 4A). We experimentally validated this observation, detecting almost undetectable levels of *PCDHGC3* mRNA in SW13 cells by RT-qPCR (Fig. 4B). These findings suggest that epigenetic silencing via promoter DNA methylation may underlie *PCDHGC3* downregulation in this model.

Targeted bisulfite sequencing confirmed high methylation levels at the *PCDHGC3* promoter in SW13 cells (Fig. 4B). To determine whether methylation directly represses gene expression, we treated SW13 cells with the DNA demethylating agent decitabine (5 μM). Strikingly, promoter methylation decreased progressively over the 6-day treatment course, accompanied by a marked, time-dependent increase in *PCDHGC3* mRNA expression. These results indicate that promoter methylation is a key mechanism of *PCDHGC3* transcriptional repression in this context.

To assess the functional impact of *PCDHGC3* upregulation, while avoiding the off-target effects of global demethylation, we transiently transfected SW13 cells with a plasmid encoding a PCDHGC3-GFP fusion protein. Fluorescence microscopy confirmed successful expression and correct subcellular localization of the fusion protein at the plasma membrane, particularly at cell-cell contact sites, consistent the typical distribution of native protocadherins (Fig. 4C).

We next investigated whether enforced *PCDHGC3* expression affects cell proliferation. Cell cycle analysis performed 48 hours post-transfection revealed a 1.5-fold increase in the G1 population and a corresponding reduction in S phase in *PCDHGC3*-expressing cells compared to controls (Fig. 4D). This pattern is consistent with G1 phase arrest or deceleration of cell cycle progression. Together, these findings support a putative tumor suppressor role for *PCDHGC3* in ACCs, consistent with its epigenetic silencing and reduced expression in aggressive tumors.

Discussion

ACC remains a highly aggressive malignancy lacking robust tissue-based biomarkers for diagnosis and risk stratification. In this study, we demonstrate that promoter hypermethylation of *PCDHGC3* reliably distinguishes ADs from ACCs and identifies a biologically aggressive subset of ACCs. These findings extend previous reports linking *PCDHGC3* methylation to tumor aggressiveness in other neuroendocrine neoplasms, including gastrointestinal neuroendocrine tumors and PPGLs [21–25], supporting its

relevance across distinct neuroendocrine contexts and suggesting a shared epigenetic mechanism of malignancy.

To our knowledge, this is the first single-locus methylation-based assay capable of distinguishing malignant from benign adrenocortical tumors with 100% specificity. This approach offers a practical alternative to complex multi-omic classifiers, many of which remain difficult to implement in routine diagnostics [14–17]. Previous studies have identified *IGF2* dysregulation as a hallmark molecular feature of ACCs [19, 29–31]. Our findings provides an additional, independent diagnostic layer that may complement histopathological assessment, particularly in ambiguous or borderline cases [3, 32]. Importantly, its discriminative performance reflects a consistent shift in methylation distribution rather than a small number of extreme cases, as demonstrated by high AUC values using continuous data and bootstrap validation. The compatibility of this assay with FFPE samples further supports its suitability for routine diagnostic workflows.

A potential concern is the relatively low absolute methylation levels observed at the diagnostic threshold. However, our findings indicate that *PCDHGC3* methylation should not be interpreted as a quantitative marker of global epigenetic burden, but rather as a binary indicator of a biologically relevant epigenetic event. In heterogeneous tumor samples, low-level methylation may reflect subclonal or cell subset–restricted promoter silencing, which can still have functional consequences. This interpretation is supported by the increased variance observed in ACCs, the consistent shift in methylation distribution, and the concordant reduction in *PCDHGC3* mRNA expression in independent datasets. Importantly, our functional data demonstrate that *PCDHGC3* is epigenetically silenced and that its re-expression induces cell cycle arrest, confirming that even limited promoter methylation can be associated with biologically meaningful gene repression. In this context, *PCDHGC3* methylation behaves as a highly specific rule-in biomarker, where the presence of methylation reliably indicates malignancy despite not being universally detectable in all tumors.

Beyond its diagnostic utility, *PCDHGC3* methylation also conveys prognostic information. Among patients with localized (ENSAT stage I–III) ACC, hypermethylation was associated with significantly shorter event-free survival, independent of established clinicopathological parameters. This finding was mirrored in the TCGA cohort, where a subset of ACCs exhibited similarly elevated *PCDHGC3* methylation levels and reduced gene expression. Notably, the association between *PCDHGC3* silencing and the CIMP-high phenotype identified by the ACC-TCGA project further supports the relevance of this locus within a broader epigenetic context of aggressive disease. Although promoter hypermethylation alone did not reach statistical significance for overall survival in TCGA, low *PCDHGC3* mRNA expression was strongly associated with poor outcome, reinforcing its biological relevance. Incorporation of *PCDHGC3* methylation into existing prognostic frameworks may therefore refine postoperative risk stratification in ACC.

Mechanistically, our *in vitro* studies demonstrate that *PCDHGC3* is epigenetically silenced by promoter methylation in ACC cells and that pharmacological demethylation restores gene expression. Re-

expression of *PCDHGC3* induced G1-phase cell cycle arrest, supporting a tumor suppressor role consistent with previous observations in other malignancies [21–25]. Together, these data provide direct functional evidence linking *PCDHGC3* methylation to transcriptional repression and tumor cell behavior and suggest that epigenetic reactivation of this locus may represent a therapeutic avenue worthy of further investigation.

Recent evidence further reinforces this role, showing that *PCDHGC3* loss drives tumor progression in clear cell renal cell carcinoma through activation of FAK signaling and downstream mTOR–HIF2α pathways, promoting proliferation, metastatic behavior, and metabolic reprogramming [26]. Although these mechanisms were not directly explored in ACC, they provide a biologically plausible framework linking *PCDHGC3* silencing to tumor aggressiveness and support the functional relevance of its epigenetic inactivation. Together, these data strengthen the interpretation that *PCDHGC3* methylation is not merely a biomarker but reflects a driver epigenetic alteration with potential therapeutic implications.

This study has limitations, including the modest number of adrenal adenomas and the enrichment for ACC cases in the primary cohort. While external validation in TCGA supports the robustness of our findings, prospective studies in independent, unselected clinical cohorts are required to define the diagnostic and prognostic utility of *PCDHGC3* methylation in routine practice.

Conclusions

PCDHGC3 promoter hypermethylation emerges as a specific, reproducible, and biologically meaningful biomarker of malignancy in adrenocortical tumors, with independent prognostic value in localized disease. Its technical feasibility in FFPE tissue and strong diagnostic performance support further validation as a clinically applicable tool for the management of ACC.

Declarations

Ethics approval and consent to participate

All samples were processed according to standardized operating procedures with approval from the corresponding Institutional Ethical and Scientific Committees. All methods were performed in accordance with relevant guidelines and regulations, and with the principles of the Declaration of Helsinki (1975), as revised in 1983.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Author Contribution

Study design: MDC. Data collection: TC, RRA, FD, YRF, IFV, FH, MTRC, IRC, JH, MAC, PJF, NV and MDC. Data analysis: TC, ASP, JSJG, NGS, RRA, JACF, NV, and MDC. Data interpretation: TC, NV, and RRA. Writing original draft: MDC. Writing-review and editing: MDC, NV, PJF. and TC. Visualization: MDC, TC, and NV.

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Data Availability

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

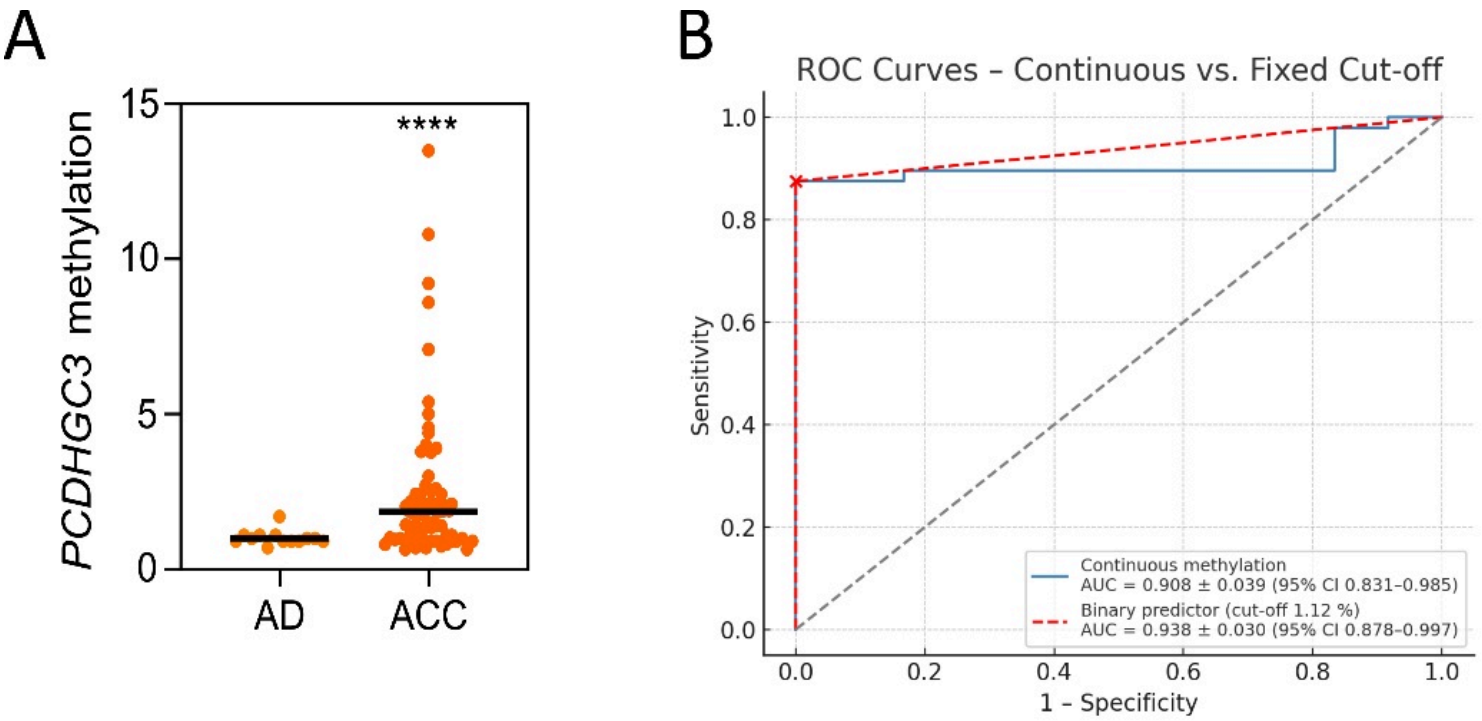


Figure 1

***PCDHGC3* promoter methylation distinguishes adrenocortical carcinoma (ACC) from adrenal adenoma (AD).** (**A**) Dot plot showing *PCDHGC3* promoter methylation levels in adrenal adenomas (AD, n = 12) and adrenocortical carcinomas (ACC, n = 48). (**B**) Receiver operating characteristic (ROC) curve comparing the diagnostic performance of continuous methylation values (blue) versus a fixed cut-off at 1.12% (red dashed line). The diagonal grey line indicates no discrimination (AUC = 0.5). The red asterisk marks the optimal cut-off point (Youden index).

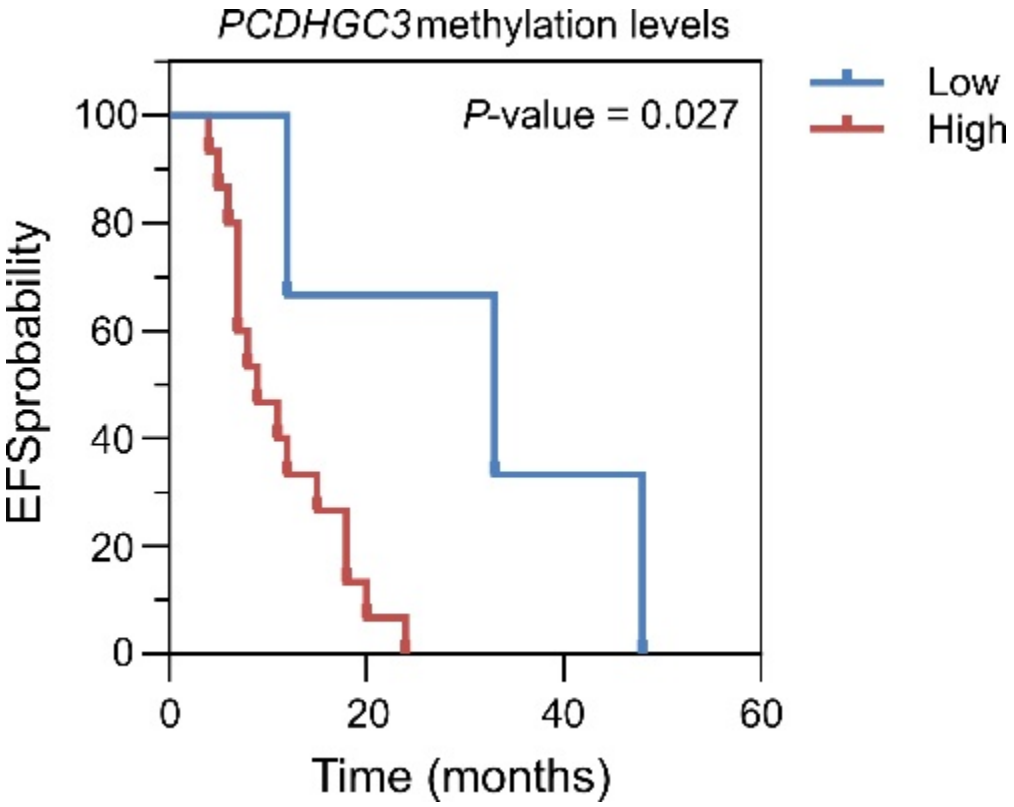


Figure 2

High *PCDHGC3* methylation levels are associated with shorter event-free survival (EFS) intervals in adrenocortical carcinoma. Kaplan–Meier analysis of EFS probability in patients with ENSAT stage I–III tumors stratified by *PCDHGC3*methylation levels. Patients with high methylation (red line) showed significantly shorter EFS compared to those with low methylation (blue line) (p = 0.027, log-rank test).

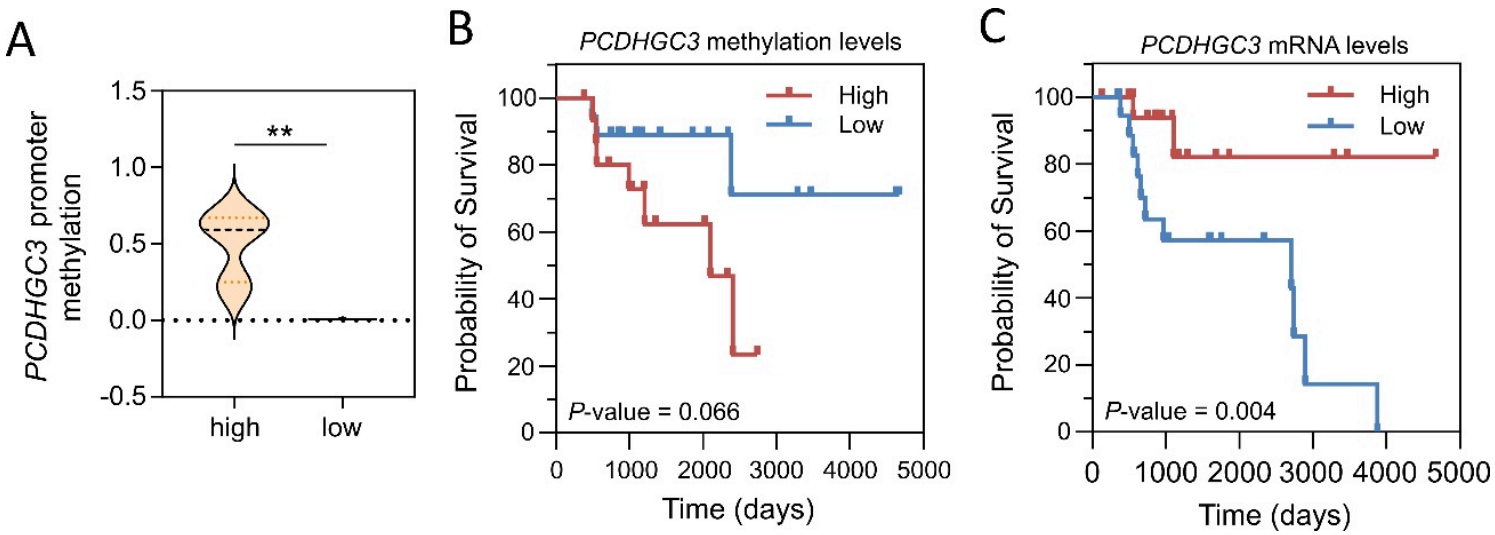


Figure 3

Prognostic significance of *PCDHGC3* methylation and expression levels in ACC. (A) Violin plot showing significantly higher *PCDHGC3* promoter methylation in a subset of ACC samples (p = 0.0031, unpaired t-

test with Welch's correction). (B, C) Kaplan–Meier analysis of OS stratified by *PCDHGC3*methylation status (B) or mRNA expression (C) levels. P values were determined by the log-rank test.

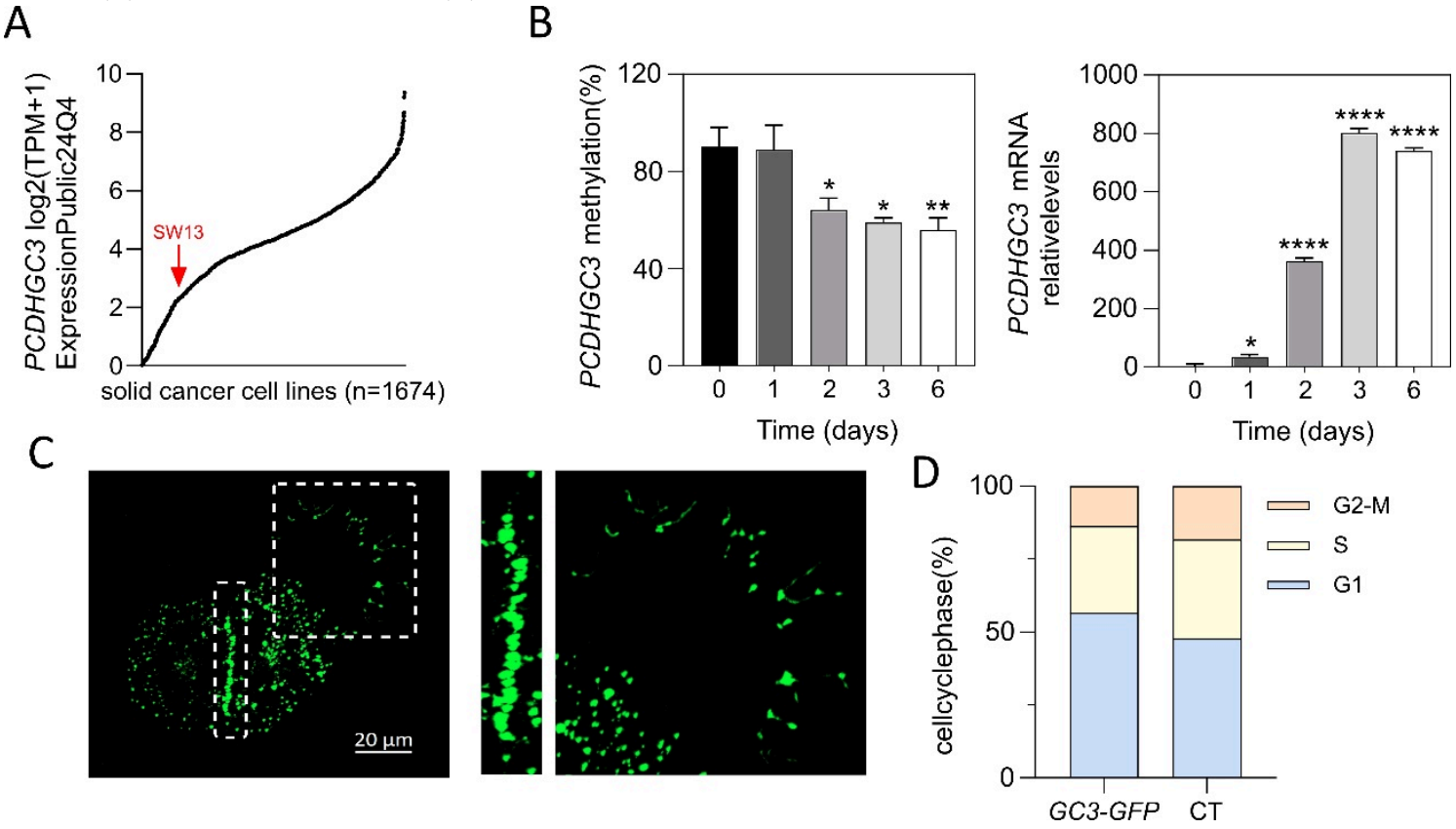


Figure 4

***PCDHGC3* is epigenetically silenced and functionally relevant in SW13 ACC cells.** (A) Ranking of *PCDHGC3*expression across 1,674 solid cancer cell lines (DepMap Expression Public 24Q4 dataset). The SW13 ACC cell line (red arrow) rank among the lowest in mRNA expression levels. (B) Left: Targeted bisulfite analysis showing progressive reduction of *PCDHGC3* promoter methylation after 1-6 days of decitabine treatment (5 M). Right: Corresponding time-dependent increase in *PCDHGC3*mRNA expression measured by qRT-PCR. Data are mean ± SD; $p < 0.05$ (*), $p < 0.01$, $P < 0.0001$ (****) versus day 0. (C) Representative fluorescence microscopy images of SW13 cells transfected with *PCDHGC3*-GFP, showing subcellular localization. Zoomed-in region highlights enrichment in cell-to-cell- contacts and plasma membrane. (D) Cell cycle distribution in *PCDHGC3*-GFP–transfected cells (GC3-GFP) versus control (CT), showing G1 accumulation.