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Clinical Research Article

Prognostic Role of Targeted Methylation Analysis in Paraffin-embedded Samples of Adrenocortical Carcinoma

Juliane Lippert,[1] Barbara Altieri,[1] Breanna Morrison,[2] Sonja Steinhauer,[1] Gabrielle Smith,[3] Antonia Lorey,[1] Hanna Urlaub,[1] Stefan Kircher,[4] Alice Sitch,[2,5] Martin Fassnacht,[1,6,7] and Cristina L. Ronchi[1,3,8]

[1]Division of Endocrinology and Diabetes, University Hospital of Wuerzburg, 97080 Wuerzburg, Germany
[2]Institute of Applied Health Research, University of Birmingham, B152TT Birmingham, UK
[3]Institute of Metabolism and System Research, University of Birmingham, B152TT Birmingham, UK
[4]Institute for Pathology, University of Wuerzburg, 97080 Wuerzburg, Germany
[5]NIHR Birmingham Biomedical Research Centre, University Hospitals Birmingham NHS Foundation Trust and University of Birmingham, B152TH Birmingham, UK
[6]Comprehensive Cancer Centre Mainfranken (CCCM), University of Wuerzburg, 97080 Wuerzburg, Germany
[7]Central Labor, University Hospital of Wuerzburg, 97080 Wuerzburg, Germany; and
[8]Centre for Endocrinology, Diabetes and Metabolism (CEDAM), Birmingham Health Partners, B152TT, Birmingham, UK

Correspondence: Cristina L. Ronchi, MD, PhD, Institute of Metabolism and System Research, College of Medical and Dental Sciences, University of Birmingham, Edgbaston B15 2TT, UK. Email: C.L.Ronchi@bham.ac.uk; or Martin Fassnacht, MD, PhD, Division of Endocrinology and Diabetes, Department of Internal Medicine 1, University Hospital, University of Wuerzburg, 97080 Wuerzburg, Germany. Email: Fassnacht_M@ukw.de.

Abstract

Context: Adrenocortical carcinoma (ACC) is a rare aggressive disease with heterogeneous prognoses. Previous studies identified hypermethylation in the promoter region of specific genes to be associated with poor clinical outcome.

Objective: Comparative analysis of promising hypermethylated genes as prognostic markers and evaluation of their added value to established clinical prognostic tools.

Design: We included 237 patients with ACCs. Tumor DNA was isolated from formalin-fixed paraffin-embedded (FFPE) samples. Targeted pyrosequencing was used to detect promoter region methylation in 5 preselected genes (*PAX5, GSTP1, PYCARD, PAX6, G0S2*). The prognostic role of hypermethylation pattern was compared with the Stage, Grade, Resection status, Age, Symptoms (S-GRAS) score. Primary endpoints were progression-free (PFS) and overall survival (OS), with disease-free (DFS) as secondary endpoint.

Results: A total of 27.9%, 13.9%, 49%, 49%, and 25.3% of cases showed hypermethylation in *PAX5, GSTP1, PYCARD, PAX6,* and *G0S2,* respectively. Hypermethylation in all individual genes—except *GSTP1*—was significantly associated with both PFS and OS—with hazard ratios (HR) between 1.4 and 2.3. However, only hypermethylation of *PAX5* remained significantly associated with OS ($P = 0.013$; HR = 1.95, 95% CI, 1.2-3.3) in multivariable analysis. A model for risk stratification was developed, combining *PAX5* methylation status and S-GRAS groups, showing improved prognostic performance compared to S-GRAS alone (Harrell's C index: OS = 0.751, PFS = 0.711, DFS = 0.688).

Conclusions: This study demonstrated that hypermethylation in *PAX5* is associated with worst clinical outcome in ACC, even after accounting for S-GRAS score. Assessing methylation in FFPE material is straightforward in the clinical setting and could be used to improve accuracy of prognostic classification, enabling the direction of personalized management.

Key Words: adrenal cancer, molecular oncology, biomarkers, prognosis, personalized medicine

Abbreviations: ACC, adrenocortical carcinoma; DFS, disease-free survival; ENSAT, European Network for the Study of Adrenocortical Tumors; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; R, resection; S-GRAS, Stage, Grade, Resection status, Age, Symptoms; TBS, targeted bisulfite sequencing

Adrenocortical carcinoma (ACC) is a rare tumor with a generally poor, but heterogeneous prognoses (5-year survival rate ranging from 13% to 80% (1, 2)). Tumor stage according to the European Network for the Study of Adrenocortical Tumors (ENSAT) classification, together with the resection (R) status of the primary tumor and the Ki67 index represent the most relevant prognostic factors (2, 3), but have a limited performance. Recent studies proposed combinations of clinical/histopathological parameters, to improve prognostic classification in patients with ACC (4-6). In particular, a large collaborative ENSAT study recently demonstrated that the Stage, Grade, Resection status, Age, Symptoms (S-GRAS) score (6), a combination of clinical (age, symptoms at diagnosis, ENSAT tumor stage) and histopathological parameters (resection status and Ki67 index), is the variable most significantly related to survival in patients with ACC.

Pan-genomic studies have identified molecular patterns associated with clinical outcome, such as a gene expression profile (ie, high *BUB1B-PINK1* levels), copy number alterations, and hypermethylation in CpG island (7, 8). Subsequently, the use of targeted methylation analysis demonstrated that hypermethylation of promoter regions in

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specific single genes—chosen among those hypermethylated in the pan-genomic methylome analysis—play a significant prognostic role. In fact, Jouinot et al demonstrated that mean methylation of 4 genes (*PAX5, GSTP1, PYCARD, PAX6*) is an independent prognostic factor of disease-free survival (DFS) and overall survival (OS) in 203 ACC cases from the ENSAT consortium (9). We have confirmed these findings in a different cohort of 107 tumor samples, whereby the methodological implementation and data analysis had been chosen with a view to future use in routine diagnostics (5). In fact, these included DNA isolated from formalin-fixed paraffin-embedded (FFPE) samples instead of fresh frozen tumors and analysis methods that do not require normalization steps. Moreover, Mohan et al showed that hypermethylation of another gene, *G0S2,* identified from the ACC-TCGA dataset, is able to distinguish a subgroup of patients with upregulated cell cycle and DNA damage response programs, and is associated with shorter DFS and OS in ACC (n = 80) (10). However, to our knowledge, the methylation of these 5 genes have never been studied simultaneously in the same cohort and their prognostic role has not been directly compared with that of clinical and histopathological parameters.

Therefore, aims of the present study were to: (1) assess and compare the prognostic role of hypermethylation in *PAX5, GSTP1, PYCARD, PAX6,* and *G0S2* as individual biomarkers and (2) investigate the advantage of methylation status as molecular marker in comparison to clinical and histopathological markers (ie, ENSAT stage, Ki67 index, and S-GRAS score) in prognostication of ACC patients. To this end, we investigated a cohort of 237 samples by using routine molecular diagnostics and easily available FFPE material.

Material and Methods

Patient Cohort and Study Design

This is a 2-center study designed and conducted in accordance with the Declaration of Helsinki. We followed the recommendations for tumor prognostic markers studies reported in REMARK (11). The study protocol was approved by local ethics committees (#88/11 at the University Hospital of Wuerzburg and HBRC 11/606 at the University of Birmingham) and written informed consent was obtained from all subjects before study enrollment.

A total of 237 patients older than age 18 years with histologically confirmed ACC, available clinical and histopathological characteristics at diagnosis, and follow-up radiological data to determine disease status and survival have been included in the present study. These enclose a cohort of 107 cases taken from a previous study (5) (cohort 1), for which we updated the survival up to October 2021 and added the methylation analysis of gene *G0S2,* plus a novel independent cohort of 130 cases (cohort 2). Demographic and baseline clinical and histopathological data for the 2 cohorts are shown in **Table 1**. For further analysis, the 2 cohorts were considered together (see statistical analysis for details).

Collection of Clinical Data

Clinical parameters such as ENSAT tumor stage, Ki67 proliferation index, resection status, age, and symptoms (related to autonomous steroid secretion or tumor mass) at the time of diagnosis were recorded for all patients. The S-GRAS score has been then calculated as previously published (6): age at diagnosis (<50 years = 0 point; ≥50 years = 1 point), hormone, tumor or systemic cancer-related symptoms at presentation (no = 0 point; yes = 1 point), ENSAT stage (1 or 2 = 0 point; 3 = 1 point; 4 = 2 points), R of primary tumor (R0 = 0 point; RX = 1 point; R1 = 2 points; R2 = 3 points), and Ki67 index (0%-9% = 0 points; 10%-19% = 1 point; ≥20% = 2 points), generating 10 S-GRAS scores. Patients were stratified into 4 groups according to the S-GRAS score as follows: S-GRAS group 0 to 1, S-GRAS group 2 to 3, S-GRAS group 4 to 5, and S-GRAS group 6 to 9.

The duration of follow-up and the clinical outcome were collected from clinical records. Primary endpoints for statistical analysis were progression-free survival (PFS) and OS, which were available for all cases. PFS was defined as the time from diagnosis to first radiological evidence of disease progress. OS was defined as the time from primary tumor resection or diagnosis to death. Additionally, as secondary endpoint, we investigated DFS that was defined as the time from complete primary tumor resection (R0) to first radiological evidence of disease relapse (n = 169). More specifically, radiological evidence of progress or relapse was defined at periodical radiological surveillance performed every 3 months by thorax-abdomen-pelvis computed tomography scan with contrast (according to current American College of Cardiology Guidelines (3)).

Tissue Sample Collection and DNA Isolation

The entire cohort included 237 paired FFPE tumor tissues and associated blood samples, used to confirm the somatic status of the methylation alterations. Among those, 197 derived from primary ACC (83% of total), 21 from local recurrence, and 19 from distant metastases (**Table 1**). The date of tumor tissue collection was between 2002 and 2015 for cohort 1 and between 2010 and 2021 for cohort 2. For all tissue samples, tumor localization and cell content was assessed in a representative FFPE slide by hematoxylin-eosin staining before DNA isolation. Tumor cell content reached a high fraction (median, 90%; range, 60%-95%). DNA was isolated from tumors with the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) and from peripheral blood with the NucleoSpin Blood L Kit (Macherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's instructions.

Methylation Analysis

Bisulfite pyrosequencing or targeted bisulfite sequencing (TBS) were used for quantitative methylation analysis of the 5 tumor suppressor genes *PAX5, PAX6, PYCARD, GSTP1*, and *G0S2*. Target regions were selected to be located within the CpG islands in the promoter regions of the genes and include, as far as possible, the regions accessible with the MLPA ME002 tumor suppressor-2 probe mix (MRC-Holland, Amsterdam, The Netherlands) used by Jouinot et al (9) and part of the region analyzed with TBS by Mohan et al (10) (**Suppl. Table 1** and **Figure S1** (12)).

Preparation of DNA samples (ie, bisulfite conversion and amplification of target regions) was conducted as already described (5). PCR amplicons were then either used for bisulfite pyrosequencing, which was also conducted as described previously (5), or for TBS. For the latter, amplicons of each patient were pooled in equal amounts, purified with Ampure XP Beads (Beckman Coulter GmbH, Krefeld, Germany) and prepared for sequencing with the Illumina DNA Prep with

Table 1. Demographic, clinical and histopathological, and molecular data for the 2 cohorts of ACC patients included in the present study

Characteristic	N	Overall, N = 237	Cohort 1, n = 107	Cohort 2, n = 130	*P* value[a]
Sex	237				0.48
F, N (%)		141 (59.49)	61 (57.01)	80 (61.54)	
M, N (%)		96 (40.51)	46 (42.99)	50 (38.46)	
Age, median (range)	237	50 (18-87)	49 (18-87)	50 (19-83)	0.77
< 50 y, N (%)		117 (49.4)	55 (51.4)	62 (52.1)	
≥ 50 y, N (%)		120 (50.6)	52 (48.6)	68 (47.7)	
ENSAT tumor stage	237				0.42
1, N (%)		14 (5.91)	4 (3.74)	10 (7.69)	
2, N (%)		106 (44.73)	51 (47.66)	55 (42.31)	
3, N (%)		71 (29.96)	29 (27.10)	42 (32.31)	
4, N (%)		45 (18.99)	23 (21.50)	22 (16.92)	
Unknown, N (%)		1 (0.42)	0	1 (0.77)	
Clinical presentation	237				0.032
Symptoms no, N (%)		109 (45.99)	41 (38.32)	68 (52.31)	
Symptoms yes, (N (%)		128 (54.01)	66 (61.68)	62 (47.69)	
Type of tumor used for analysis	237				0.96
Metastasis		19.00 (8.02%)	9.00 (8.41%)	10.00 (7.69%)	
Primary		197.00 (83.12%)	89.00 (83.18%)	108.00 (83.08%)	
Recurrence		21.00 (8.86%)	9.00 (8.41%)	12.00 (9.23%)	
Resection status	237				0.038
R0, N (%)		169 (71.31)	74 (69.16)	95 (73.08)	
RX, N (%)		22 (9.28)	16 (14.95)	6 (4.62)	
R1, N (%)		18 (7.59)	5 (4.67)	13 (10.00)	
R2, N (%)		18 (7.59)	9 (8.41)	9 (6.92)	
Unknown, N (%)		10 (4.22)	3 (2.80)	7 (5.38)	
Ki67 index	237				0.019
0-9, N (%)		56 (23.63)	31 (28.97)	25 (19.23)	
10-19, N (%)		57 (24.05)	33 (30.84)	24 (18.46)	
≥20, N (%)		113 (47.68)	43 (40.19)	70 (53.85)	
Unknown, N (%)		11 (4.64)		11 (8.46)	
S-GRAS groups	237				0.66
S-GRAS group 0-1, N (%)		37 (15.61)	19 (17.76)	18 (13.85)	
S-GRAS group 2-3, N (%)		100 (42.19)	43 (40.19)	57 (43.85)	
S-GRAS group 4-5, N (%)		67 (28.27)	28 (26.17)	39 (30.00)	
S-GRAS group 6-9, N (%)		33 (13.92)	17 (15.89)	16 (12.31)	
Methylation score					
Hypermethylated *G0S2*, N (%)		60 (25.32)	24 (22.43)	36 (27.69)	0.35
Hypermethylated *GSTP1*, N (%)		33 (13.92)	12 (11.21)	21 (16.15)	0.27
Hypermethylated *PAX5*, N (%)		66 (27.85)	22 (20.56)	44 (33.85)	0.023
Hypermethylated *PAX6*, N (%)		116 (48.95)	50 (46.73)	66 (50.77)	0.54
Hypermethylated *PYCARD*, N (%)		116 (48.95)	47 (43.93)	69 (53.08)	0.16

Methylation score, methylation status per gene was calculated by averaging the methylation levels of all corresponding CpGs, which, in turn, were calculated from the ratio of methylated cytosine to total coverage at the appropriate position. Methylation status > 25% was classified as hypermethylated.
Abbreviations: ACC, adrenocortical carcinoma; ENSAT, European Network for the Study of Adrenocortical Tumors; F, female; M, male; S-GRAS, Stage, Grade, Resection status, Age, Symptoms.
[a]*P* values indicate comparability of the variables between the 2 cohorts. Age was analyzed using Mann-Whitney tests, ENSAT and resection status were analyzed using Fisher test, and all other variables were analyzed using χ^2 tests.

Enrichment Kit (Illumina Inc, San Diego, CA, USA), according to the manufacturers protocol. Samples were sequenced on a MiSeq (Illumina Inc). Raw data were aligned and analyzed with GensearchNGS (Phenosystems S.A., Belgium). Methylation level of each CpG was calculated from the ratio of methylated cytosine to total coverage at the appropriate position. The methylation status of a sample for the promoter region of each gene was determined by averaging the methylation levels of the corresponding CpGs.

All 5 genes were estimated as hypomethylated with a methylation status of ≤ 25% and hypermethylated with a methylation status > 25% (cutoff from Jouinot et al (9)).

The comparability of the data obtained with the 2 different methods had been tested in advance on 5 randomly selected samples for all 5 genes. Except for 1 gene in 1 sample, the methylation data were comparable in means of methylation status (ie, hypo- or hypermethylation). On average, a change in methylation status of 6.4 percentage points was observed (data not shown).

Moreover, in a subgroup of 9 patients, we compared the methylation status between DNA isolated from fresh frozen tissue ([isolated with GenElute Mammalian Genomic DNA Miniprep Kit [Sigma Aldrich—Merck KGaA, Darmstadt, Germany] or Maxwell RSC Blood Kit [Promega, Walldorf, Germany] according to the manufacturer's protocols) and DNA isolated from FFPE tissue gained from the same tumor. By comparing methylation status classified as hypo- or hypermethylated, we found superimposable findings (ie, no differences in methylation status for *PYCARD* and *PAX6*, and a discrepancy in only 1 of 9 patients for *PAX5*, and 2 of 9 samples for *G0S2* and *GSTP1)* (data not shown).

Statistical Analysis

Descriptive statistics were produced to compare the 2 cohorts and the overall data (Table 1). Mann-Whitney *U* test and χ^2 tests were used, as appropriate, to compare baseline data, between the 2 cohorts. Further analyses included study cohort as a covariate to enable adjustment.

Kaplan-Meier plots were used to investigate the proportional hazards assumption and to display the unadjusted survival curves for survival outcomes. Hazard ratio (HR), 95% CI, and *P* values were reported for each of the 3 survival outcomes (OS, PFS, and DFS).

Cox survival models were fitted for each of the 3 outcomes (OS, PFS, and DFS). Cox proportional hazards models were fitted using methylation data of each of the 5 genes (*G0S2*, *GSTP1*, *PAX5, PAX6*, *PYCARD*) and S-GRAS score separately as an independent variable; a further model included all individual 5 genes and S-GRAS score to see if the information about methylation status improved the model. Finally, we performed an exploratory analysis, with and without the inclusion of 2-way interaction terms between the individual genes and the S-GRAS scores in the model, with backwards selection used to reduce the variables.

The discriminative performance of the ENSAT tumor stage, Ki67 index, S-GRAS groups, and methylation status on survival models were compared using the Harrell's Concordance index (C-index) (13).

For Fig. 1, samples are sorted according to PFS divided into poor (PFS < 6 months), intermediate (PFS 7-11 months), good (PFS ≥ 12 months) or still not applicable prognosis.

Statistical analysis was done using R Statistics (version: 4.1.2). A *P* value < 0.05 was considered statistically significant.

Results

Clinical Characteristics and Methylation Status

The baseline clinical and histopathological characteristics and methylation status of the patients with ACC in the 2 cohorts are listed in Table 1. In brief, 54.0% of patients presented with symptoms, 50.6% were diagnosed at an early stage (ie, ENSAT stage 1-2) and in 71.3% of cases the tumor was completely resected. In nearly half of the tumors, the Ki67 index was ≥ 20%. Specifically, clinical presentation, R status, and Ki67 were significantly different between the 2 cohorts (*P* = 0.032, *P* = 0.038, and *P* = 0.019, respectively). According to the S-GRAS score, 37 patients were classified as S-GRAS group 0 to 1 (15.6%), 100 as S-GRAS group 2 to 3 (42.2%), 67 as S-GRAS group 4 to 5 (28.3%), and 33 as S-GRAS group 6 to 9 (13.9%), respectively.

Hypermethylation of the promotor region of *PAX5* was found in 66 samples (27.9%; median methylation, 12.5%; range, 0.5%-97.5%), *GSTP1* in 33 samples (13.9%; median methylation, 2.2%; range, 0%-73.8%), *PYCARD* in 116 samples (49%; median methylation, 23.3%; range, 0.5%-94.3%), *PAX6* in 116 samples (49%; median methylation; 24.7%; range, 0.7%-97.0%), and *G0S2* in 60 samples (25.3%; median methylation, 4.0%; range, 0%-94.4%). Samples in cohort 2 were significantly more likely to have hypermethylation of *PAX5* (*P* = 0.023).

Methylation data of each gene for each sample classified as hypo- or hypermethylated and matched clinical and histopathological data as well as clinical outcome, are provided as a heatmap in Fig. 1. For *G0S2*, *GSTP1*, *PAX5*, and *PAX6*, the percentage of samples that are hypermethylated increases significantly from good to bad prognosis group (*G0S2*: 17%, 29%, and 36% (*P* = 0.01); *GSTP1*: 7%, 11%, and 23% (*P* = 0.005); *PAX5*: 21%, 18%, and 42% (*P* = 0.002); and *PAX6*: 39%, 47%, and 65% (*P* = 0.0015)).

Prognostic Role of Methylation Pattern in Promoter Regions of Individual Genes

We analyzed the relationship between methylation status and survival. Details for the entire cohort of 237 cases are shown in Fig. 2 and Table 2. Specifically, CpG island hypermethylation of all 5 genes showed significant prognostic impact on PFS at univariate analysis with HR ranging from 1.405 to 1.882. Hypermethylation of genes *G0S2*, *PAX5*, *PAX6,* and *PYCARD* was also significantly related to OS (with HR from 1.568 to 2.256), whereas hypermethylation of *GSTP1* was not. Finally, hypermethylation of all genes, except *PYCARD*, was also a significant prognostic factor of DFS.

Prognostic Role of Clinical and Histopathological Parameters

The 2 prognostic markers routinely used for prognostication of ACC—Ki67 index and ENSAT stage—were significantly associated with survival. For instance, patients with a tumor having a Ki67 ≥ 20 or diagnosed at ENSAT stage 4 had significantly shorter PFS (HR = 3.52 [95% CI, 2.33-5.32] and 3.77 [95% CI, 2.58-5.53]), OS (HR = 3.98 [2.32-6.83] and 3.16 [2.00-4.98]), and DFS (HR = 3.28 [2.00-5.37] and 4.82 [2.64-8.78]) compared with patients with Ki67 = 0-9 and ENSAT stage 1 and 2, respectively. The corresponding C-indices ranged between 0.646 and 0.679 and 0.628 and0.669 (**Figure S2** (12) and Table 2).

The S-GRAS score (6) group 6 to 9 was significantly related to OS and PFS when compared with S-GRAS group 0 to 1 (*P* < 0.001, HR = 8.79 [4.05-19.10], and *P* < 0.001, HR = 7.09 [3.92-12.80], respectively (Table 2)). The DFS model also demonstrated a significant relationship with S-GRAS group 6 to 9 having an HR of 22.46 when compared with the 0 to 1 group (*P* < 0.001; 95% CI, 4.85-104.02). Finally, the C-index was higher for the S-GRAS (having the best single variable model performance) compared with Ki67 index and ENSAT stage (Table 2 and Fig. 3).

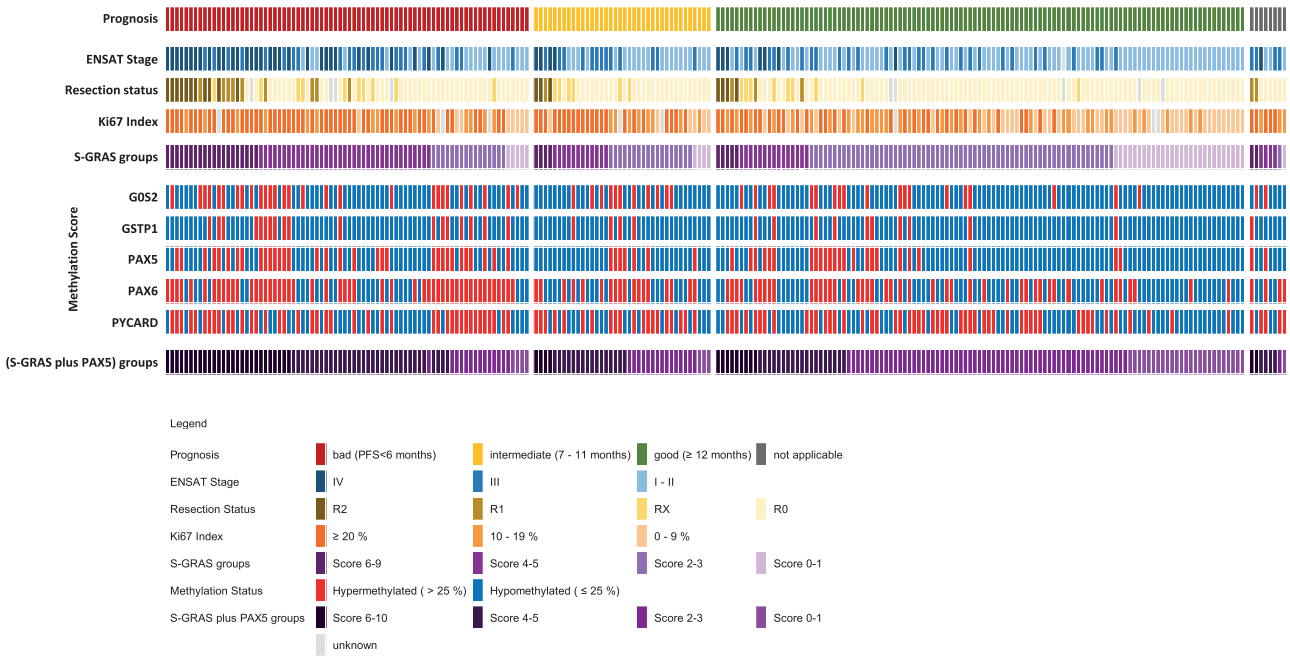

Figure 1. Heatmap of clinical and histopathological data and methylation data for patients with adrenocortical carcinoma (ACC, n = 237). For this figure, patients are sorted according to progression-free survival (PFS) divided into poor (PFS < 6 months), intermediate (PFS 7-11 months), good (PFS ≥ 12 months), or still not applicable prognosis. Subgroups are further sorted according to S-GRAS score grouping.

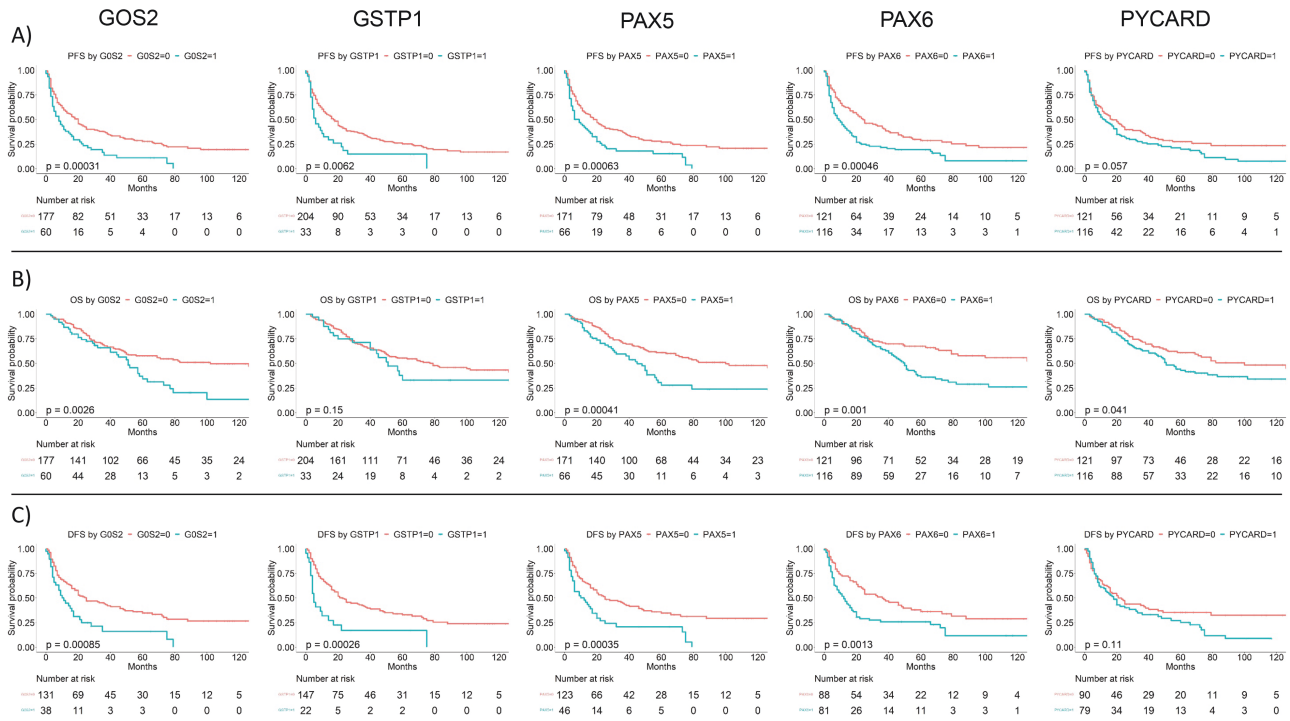

Figure 2. Unadjusted univariate (Kaplan-Meier) survival curves for patients with adrenocortical carcinoma (ACC) for methylation status of 5 investigated individual genes: *G0S2*, *GSTP1*, *PAX5*, *PAX6*, *PYCARD*. (A) Progression-free survival (PFS), (B) overall survival (OS), and (C) disease-free survival (DFS).

Prognostic Role of Methylation Pattern Compared With and Combined With Clinical Parameters

We used a regression model with interaction effects to investigate if any of the genes remained significantly related to survival after adjustment against S-GRAS score. At multivariable analysis, the *PAX5* gene was the only one whose methylation status was still significantly associated (for OS only) ($P = 0.013$, adjusted HR = 1.95; 95% CI, 1.15-3.30; details in Table 3). The PFS model was the only model that had a significant interaction effect (ie, an interaction between *PAX5* and S-GRAS group 4-5). In the reduced model, without interaction terms, including only

Table 2. Prognostic role of hypermethylation pattern in 5 preselected genes and clinical/pathological parameters. Univariable survival analysis for progression free survival (PFS), overall survival (OS), and disease-free survival (DFS)

	HR (95%CI)	*P* value	Harrell's C index
Hypermethylation pattern			
G0S2-PFS	1.856 (1.336-2.578)	< 0.001	0.592
G0S2-OS	1.825 (1.229-2.711)	0.003	0.583
G0S2-DFS	2.058 (1.355-3.124)	0.001	0.591
GSTP1-PFS	1.863 (1.234-2.813)	0.003	0.579
GSTP1-OS	1.548 (0.941-2.546)	0.086	0.572
GSTP1-DFS	2.524 (1.531-4.161)	< 0.001	0.591
PAX5-PFS	1.882 (1.358-2.607)	< 0.001	0.593
PAX5-OS	2.256 (1.52-3.348)	< 0.001	0.611
PAX5-DFS	2.122 (1.427-3.154)	< 0.001	0.602
PAX6-PFS	1.745 (1.297-2.347)	< 0.001	0.607
PAX6-OS	1.863 (1.282-2.707)	0.001	0.594
PAX6-DFS	1.871 (1.293-2.708)	< 0.001	0.612
PYCARD-PFS	1.405 (1.044-1.891)	0.025	0.558
PYCARD-OS	1.568 (1.083-2.269)	0.017	0.590
PYCARD-DFS	1.390 (0.960-2.012)	0.081	0.539
Clinical and histopathological characteristics			
Ki67 10-19-PFS	1.408 (0.889-2.231)	0.145	0.662
Ki67 ≥ 20-PFS	3.522 (2.332-5.319)	< 0.001	-
Ki67 10-19-OS	1.514 (0.8085-2.836)	0.195	0.679
Ki67 ≥ 20-OS	3.978 (2.318-6.826)	< 0.001	-
Ki67 10-19-DFS	1.284 (0.744-2.216)	0.369	0.646
Ki67 ≥ 20-DFS	3.277 (2.000-5.370)	< 0.001	-
ENSAT stage 3-PFS	1.534 (1.088-2.163)	0.0146	0.665
ENSAT stage 4-PFS	3.774 (2.577-5.527)	< 0.001	-
ENSAT stage 3-OS	1.920 (1.247-2.957)	0.003	0.669
ENSAT stage 4-OS	3.158 (2.003-4.979)	< 0.001	-
ENSAT stage 3-DFS	1.4665 (0.9746-2.207)	0.0663	0.628
ENSAT stage 4-DFS	4.8174 (2.643-8.780)	< 0.001	-
S-GRAS group 2-3-PFS	1.814 (1.097-3.000)	0.020	0.698
S-GRAS group 4-5-PFS	5.091 (2.981-8.697)	< 0.001	-
S-GRAS group 6-9-PFS	7.088 (3.923-12.802)	< 0.001	-
S-GRAS group 2-3-OS	1.977 (0.951-4.109)	0.0680	0.729
S-GRAS group 4-5-OS	4.962 (2.379-10.349)	< 0.001	-
S-GRAS group 6-9-OS	8.790 (4.046-19.096)	< 0.001	-
S-GRAS group 2-3-DFS	1.948 (1.125-3.374)	0.0174	0.664
S-GRAS group 4-5-DFS	5.781 (3.086-10.83)	< 0.001	-
S-GRAS group 6-9-DFS	22.455 (4.848-104.016)	< 0.001	-

Cohort *P* value = if cohort is significantly related to outcome variable when controlling for the variable; variable *P* value = if variable is significantly related to outcome variable when controlling for the cohort effect.
Abbreviation: HR, hazard ratio.

PAX5 and S-GRAS grouping, the independent prognostic role of hypermethylated *PAX5* was even more evident, being significant for all outcomes: OS (adjusted HR = 2.08; 95% CI, 1.39-3.12), PFS (adjusted HR = 1.67; 95% CI, 1.19-2.35), and DFS (adjusted HR = 1.92; 95% CI, 1.27-2.88) (**Table 3**).

Of note, the comparison among C-indices of the different prediction models shows an improved goodness-of-fit measure

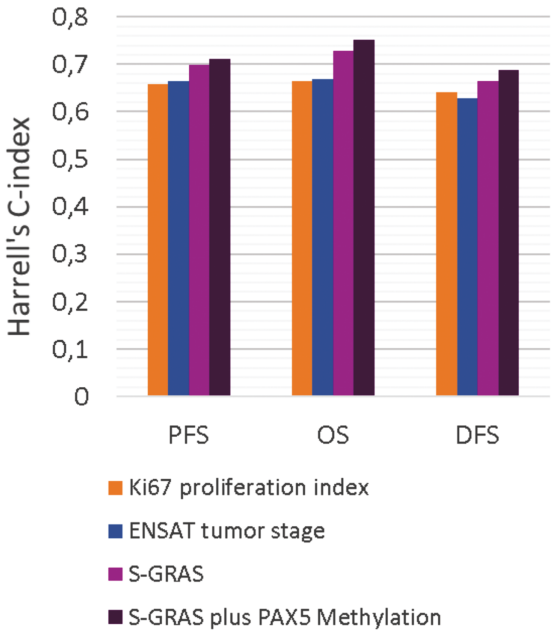

Figure 3. Comparison of Harrell's C-index for Ki67 proliferation index, ENSAT tumor stage, S-GRAS, and the combination of S-GRAS with methylation status of *PAX5*. Progression-free survival (PFS), overall survival (OS), and disease-free survival (DFS) are shown.

for S-GRAS grouping plus methylation status of *PAX5* (C index: PFS = 0.711, OS = 0.751, DFS = 0.688), compared with S-GRAS groups (C-index: PFS = 0.698, OS = 0.729, DFS = 0.664), ENSAT stage (C-index: PFS = 0.665, OS = 0.669, DFS = 0.628), and Ki67 score (C index: PFS = 0.662, OS = 0.679, DFS = 0.646) (**Fig. 3**).

Discussion

Here, we describe the methylation status of 5 genes previously reported to be associated with clinical outcome in ACC (*G0S2*, *GSTP1*, *PAX5*, *PAX6*, *PYCARD)* (9, 10) in a large and well-characterized cohort of patients (n = 237). We used methods easily applicable in the clinical practice starting from FFPE tissue material. Of note, we validated for the first time the prognostic role of hypermethylation in selected genes against the most accurate clinical-pathological classification (ie, the S-GRAS grouping stratification (5, 6)) to prioritize their use in clinical practice.

The relevance of methylation alterations for gene regulation and cancer development is well known since the 1980s (14). Methylation differences and their role in tumorigenesis in adrenocortical tumors were first reported in context with the imprinted 11p15 locus (15). Genome-wide methylation analysis revealed that ACCs can be subclassified according to CpG island methylation phenotype, which is associated to survival (7, 16). The targeted assessment of those subclasses was first described by Jouinot et al (9) that identified the hypermethylation of 4 specific genes as significantly associated with survival in ACC (*GSTP1*, *PAX5*, *PAX6*, *PYCARD)*. More recently, we further adapted targeted methylation analysis of these 4 genes for DNA isolated from FFPE tissue samples for a possible prospective use in routine diagnostics (5). Namely, we used pyrosequencing, an easily applicable method that allows to generate absolute values without

Table 3. Multivariable analysis by Cox survival model for S-GRAS score groups and hypermethylation pattern for 5 selected genes (upper panel) and for exploratory analysis without interaction terms (*PAX5* only, bottom panel)

	PFS HR (95% CI)	*P* value	OS HR (95% CI)	*P* value	DFS HR (95% CI)	*P* value
G0S2	1.117 (0.734-1.701)	0.605	1.143 (0.679-1.923)	0.615	1.079 (0.621-1.875)	0.787
GSTP1	0.995 (0.581-1.704)	0.986	0.700 (0.351-1.399)	0.313	1.674 (0.841-3.329)	0.142
PAX5	1.350 (0.881-2.068)	0.169	1.946 (1.151-3.288)	0.013	1.304 (0.742-2.292)	0.357
PAX6	1.303 (0.911-1.863)	0.148	1.215 (0.774-1.907)	0.397	1.352 (0.869-2.104)	0.181
PYCARD	1.146 (0.818-1.605)	0.429	1.354 (0.897-2.042)	0.149	1.027 (0.686-1.537)	0.897
S-GRAS group 2-3	1.574 (0.934-2.651)	0.089	1.668 (0.795-3.503)	0.176	1.642 (0.927-2.906)	0.089
S-GRAS group 4-5	4.386 (2.525-7.619)	<0.001	4.482 (2.135-9.408)	<0.001	5.263 (2.74-10.11)	<0.001
S-GRAS group 6-9	5.989 (3.256-11.018)	<0.001	6.798 (3.054-15.132)	<0.001	13.345 (2.569-69.317)	0.002
Cohort	0.608 (0.443-0.836)	0.002	0.513 (0.341-0.771)	0.001	0.653 (0.443-0.961)	0.031
	PFS HR (95% CI)		**OS HR (95% CI)**		**DFS HR (95% CI)**	
PAX5	1.675 (1.195-2.348)	0.003	2.079 (1.386-3.118)	<0.001	1.915 (1.273-2.881)	0.002
S-GRAS group 2-3	1.671 (1.005-2.780)	0.048	1.835 (0.879-3.830)	0.106	1.706 (0.976-2.983)	0.061
S-GRAS group 4-5	4.529 (2.626-7.809)	<0.001	4.599 (2.195-9.636)	<0.001	5.100 (2.694-9.655)	<0.001
S-GRAS group 6-9	6.784 (3.746-12.287)	<0.001	7.989 (3.659-17.446)	<0.001	23.754 (5.132-109.958)	<0.001
Cohort	0.608 (0.443-0.836)	0.002	0.513 (0.341-0.771)	0.001	0.653 (0.443-0.961)	0.031
Harrell's C-index[a]	0.711		0.751		0.688	

Abbreviations: DFS, disease-free survival; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.
[a]Calculated for the bottom panel.

complex normalization steps (as needed for methylation-specific MLPA) (9). Moreover, it has been shown in a small cohort that hypermethylation in selected genes represent a reliable biomarker, stable over time (ie, from primary tumors to recurrences and distant metastases) and unaffected by previous therapies (17).

In the present study, we investigated the methylation status of the 4 genes reported by the French group (*GSTP1*, *PAX5*, *PAX6*, *PYCARD)* (9) and 1 additional gene previously proposed by Mohan et al (*G0S2*) (10). First, we confirmed that hypermethylation in all the 5 genes was significantly associated with worst clinical outcome at univariable analysis. Second, we adjusted the impact of hypermethylation on survival including in our model the most accurate clinicopathological classification (ie, the S-GRAS score grouping) (6). Here, we demonstrated that only hypermethylated *PAX5* remained significant as independent prognostic marker for OS. Importantly, the comparison of the discriminatory power by Harrell's C-index, showed the best performance by a combination of S-GRAS groups and *PAX5* methylation status, followed by S-GRAS alone, Ki67 index, and ENSAT stage. This finding indicates that adding *PAX5* methylation status to S-GRAS risk stratification could further improve the accuracy of initial prognostic classification of patients with ACC. This is of important clinical relevance for decision making because it could allow clinicians to better select patients for adjuvant and/or more aggressive treatment. For instance, patients classified as "good prognosis" based on clinical parameters (ie, within S-GRAS group 0-1 or 2-3), but positive for *PAX5* methylation (11% and 30%, respectively) might benefit from adjuvant treatment. This remains to be verified by further studies.

Importantly, considering that hypermethylation in 1 or more of the investigated genes represents surrogate of an overall hypermethylation pattern (9, 10), this is not only relevant for prognostic stratification of patients with ACC, but theoretically also as a potentially druggable molecular event. In fact, the design of therapeutic strategies involving drugs targeting epigenetic events is a growing field. Epigenetic drugs are small molecules that act on the enzymes that maintain and establish epigenetic modifications. Several therapeutics targeting the epigenome are already approved by the US Food and Drug Administration. Though their use is specified for leukemias and lymphomas (summarized by Jones et al (18)), so far, the use in solid tumors also in combination with other conventional therapies is under investigation (reviewed by Morel et al (19)).

The present study has some limitations. First, breaking participants down by S-GRAS group and hypermethylated gene presence may result in a smaller than needed sample size, making it difficult to determine if gene importance varies based on S-GRAS score. Future larger studies should consider the possibility of these interaction effects. Second, as with all retrospective studies, the impact of pharmacological treatments is not taken into account for OS and PFS. Third, other molecular markers previously proposed to be potentially associated with survival, such as gene expression of *BUB1B-PINK1* (20, 21), somatic copy number alteration patterns, single nucleotide variants, or small insertions and deletions in genes of ß-catenin or TP53 signaling pathway (7, 8, 22), have not been considered. These are, however, still not straightforward analyses to be proposed for clinical practice.

In conclusion, we showed that adding *PAX5* methylation status to S-GRAS grouping may improve the prognostic classification of patients with ACC, compared with only using S-GRAS score. Targeted pyrosequencing of 1 gene in FFPE tissue material represents a straight forward, cheap, and robust methodology applicable in specialized centers that could be easily implemented in clinical practice. Therefore, we propose to add targeted methylation analysis to S-GRAS stratification to better discriminate patients with different clinical outcomes, thus enabling a more personalized medical management (ie, choice of adjuvant treatment).

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Disclosures

The authors declare no potential conflict of interest.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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