

# Methylation of *IGF2* regulatory regions to diagnose adrenocortical carcinomas

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## Abstract

Adrenocortical carcinoma (ACC) is a rare malignancy with a poor prognosis. Discrimination of ACCs from adrenocortical adenomas (ACAs) is challenging on both imaging and histopathological grounds. High *IGF2* expression is associated with malignancy, but shows large variability. In this study, we investigate whether specific methylation patterns of *IGF2* regulatory regions could serve as a valuable biomarker in distinguishing ACCs from ACAs. Pyrosequencing was used to analyse methylation percentages in DMR0, DMR2, imprinting control region (ICR) (consisting of CTCF3 and CTCF6) and the *H19* promoter. Expression of *IGF2* and *H19* mRNA was assessed by real-time quantitative PCR. Analyses were performed in 24 ACCs, 14 ACAs and 11 normal adrenals. Using receiver operating characteristic (ROC) analysis, we evaluated which regions showed the best predictive value for diagnosis of ACC and determined the diagnostic accuracy of these regions. In ACCs, the DMR0, CTCF3, CTCF6 and the *H19* promoter were positively correlated with *IGF2* mRNA expression ( $P < 0.05$ ). Methylation in the most discriminating regions distinguished ACCs from ACAs with a sensitivity of 96%, specificity of 100% and an area under the curve (AUC) of  $0.997 \pm 0.005$ . Our findings were validated in an independent cohort of 9 ACCs and 13 ACAs, resulting in a sensitivity of 89% and a specificity of 92%. Thus, methylation patterns of *IGF2* regulatory regions can discriminate ACCs from ACAs with high diagnostic accuracy. This proposed test may become the first objective diagnostic tool to assess malignancy in adrenal tumours and facilitate the choice of therapeutic strategies in this group of patients.

## Key Words

- ▶ adrenocortical carcinoma
- ▶ biomarker
- ▶ DNA methylation
- ▶ epigenetics
- ▶ *IGF2*

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## Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy, with an estimated incidence of 0.7–2.0 cases per million population each year (Kebebew *et al.* 2006, Golden *et al.* 2009, Fassnacht *et al.* 2013, Kerkhofs *et al.* 2013).

The prognosis is poor, with overall 5-year survival rates of 16–44% (Fassnacht *et al.* 2010). The likelihood of malignancy of an adrenal mass is based on the CT/MRI phenotype (e.g. size, Hounsfield units, contrast washout)

and is decisive for surgery (Lacroix 2010). However, the discrimination of ACCs from adrenocortical adenomas (ACAs) still remains challenging on both imaging and postoperative histopathological grounds. The current pathological scoring system (Weiss score) is limited by a lack of reproducibility and lacks diagnostic accuracy in so-called 'borderline malignant' tumours with a Weiss score of 2 or 3 (Lau & Weiss 2009, de Krijger & Papathomas 2012). The assessment of malignant potential in adrenocortical tumour variants and/or paediatric adrenocortical neoplasms can be challenging and requires an experienced histopathologist; accordingly, applying the Weiss scoring system could tilt the diagnosis toward malignancy in oncocytic tumours and/or tumours in the paediatric setting (Dehner & Hill 2009, Papotti et al. 2014), whereas underdiagnosis could possibly be the case when confronted with myxoid variants (Lau & Weiss 2009, de Krijger & Papathomas 2012, Papotti et al. 2014). A biomarker that determines malignancy in adrenal tumours would be an important improvement in diagnostic certainty that can underpin therapeutic strategies such as adjuvant mitotane treatment and intensity and duration of follow-up (Young 2007).

The adrenal gland produces various growth factors, including the insulin-like growth factor 2 (*IGF2*). *IGF2* is an imprinted gene primarily expressed from the paternal allele (Ohlsson et al. 1994) and is correlated with malignancy in adrenal tumours (Erickson et al. 2001, Giordano et al. 2003, de Fraipont et al. 2005, Almeida et al. 2008, Wang et al. 2014). However, there is a large variability in *IGF2* expression and *IGF2* expression levels are not fully discriminative for a distinction between ACC and ACA (Schmitt et al. 2006, Wang et al. 2014). By the

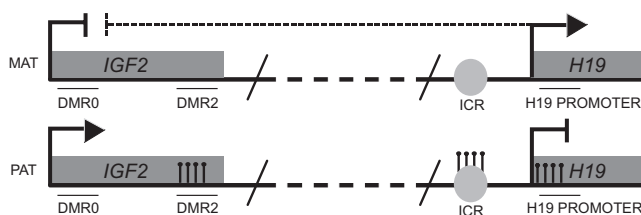
concept of DNA methylation, several factors are supposed to regulate *IGF2* expression (Fig. 1).

*H19*, a gene whose transcript is not translated, is reciprocally imprinted with *IGF2* (Rachmilewitz et al. 1992, Zhang & Tycko 1992). Low expression of the *H19* gene may play a role in the development of ACCs in two different ways: *H19* per se is supposed to be involved in tumour suppression and methylation of the *H19* gene regulates mRNA expression of *H19* and *IGF2* (Hao et al. 1993). Previous research revealed a higher mean degree of methylation in the *H19* promoter in ACCs compared with ACAs (Gao et al. 2002), but again with a significant overlap between both tumour entities.

Also differentially methylated regions (DMRs) play a role in the regulation of *IGF2* expression. DMRs are regions in the genome with high concentration of CpGs controlling imprinting. In other types of cancer, it has been proven that hypomethylation of the DMR0 correlates with *IGF2* loss of imprinting (LOI) (Baba et al. 2010). Previous research also suggests that hypomethylation of DMR2 is associated with ACCs and increased *IGF2* expression (Barlaskar 2011).

The imprinting control region (ICR) is another element that contributes to regulation of *IGF2*. Methylation of this region determines whether *IGF2* or *H19* is expressed from the allele (Dejeux et al. 2009), as a result of binding of the methylation-specific CTCF protein to this region (Herold et al. 2012). Two of the key CTCF-binding sites are the third (CTCF3) and sixth (CTCF6) sites (Boissonnas et al. 2010).

In this study, DNA methylation is assessed by pyrosequencing, the most sensitive and accurate method to detect methylation at a single CpG (Quillien et al. 2012). To the best of our knowledge, we analyse for the first time methylation in a number of different *IGF2* regulatory regions in benign and malignant adrenal tumours. We aimed to identify specific patterns correlating with malignancy in adrenal tumours. As such, our main research aim is to evaluate whether the methylation patterns of *IGF2* regulatory regions, individually or combined, could serve as a valuable biomarker in distinguishing ACCs from ACAs. Finally, to demonstrate that methylation is involved in regulation of *IGF2* expression in ACC, we examined the effect of demethylation in ACC cell lines.



**Figure 1**

Overview of the imprinted region of the *IGF2* gene and *H19* gene on the maternal and paternal allele in the normal situation. Lines with dots represent known sites of DNA methylation. (arrow) expression of the gene. (stub-end arrow) repression of the gene. Methylation status of the DMR0 remains unclear. Figure is not at scale. DMR0, differentially methylated region 0; DMR2, differentially methylated region 2; ICR, imprinting control region, consisting of CTCF3 and CTCF6. Data from Tabano et al. (2010).

## Methods

### Adrenocortical tissues

We obtained normal adrenals and adrenocortical tumours from patients during surgeries performed

at the Department of Surgery, Rotterdam, Erasmus MC (EMC) between May 1995 and March 2014. We embedded the specimens in Tissue-Tek after resection and stored them at  $-80^{\circ}\text{C}$  until analysis. All normal adrenals were collected during nephrectomy and confirmed by the pathologist as being normal. We randomly selected ACAs based on availability of tumour tissue in the past 7 years. We excluded nonprimary ACCs ( $n=22$ ), ACCs with insufficient DNA or RNA yield ( $n=7$ ), or tumours defined as 'borderline malignant' by the pathologist ( $n=2$ ; [Supplementary Fig. 1](#), see section on [supplementary data](#) given at the end of this article). We collected medical history information and tumour characteristics from electronic patient records ([Table 1](#)). Follow-up data were updated from the Pathological Anatomical National Automated Archives (PALGA). An independent pathologist collected all data for pathological features, including the Weiss score, the currently used pathological scoring system for determining malignancy in adrenocortical tumours ([Lau & Weiss 2009](#)). A cutoff of 3 criteria or more present in the tumour was considered malignant, although only tumours with a clear pathological diagnosis were included in the analysis. We used the ENSAT classification to stage the tumours ([Lughezzani et al. 2010](#)). To validate our findings in an independent cohort, we used a series of frozen specimens from the

VU University Medical Center (VUMC), Amsterdam, the Netherlands. DNA isolation and methylation analyses were performed at the EMC. The Weiss score was independently determined both at the VUMC and at the EMC as a central review. The study was conducted under the guidelines that had been approved by the Medical Ethics Committee of the Erasmus Medical Center.

### Processing of adrenocortical tissues

We used several cryostat sections of  $20\mu\text{m}$  to isolate RNA and DNA from Tissue-Tek embedded tissues and  $5\mu\text{m}$  sequential cryostat sections for haematoxylin-eosin (H&E) staining in order to confirm that we used representative tissue specimens. We considered a tissue as representative when it contained at least 80% of tumour cells in case of ACA or ACC, or when a clear normal adrenal structure was visible at microscopic evaluation.

### Cell culture

We used three available human ACC cell lines: H295R and SW13 were obtained from the American Type Culture Collection and from ECACC (Salisbury, Wiltshire, UK), respectively. HAC15 cells were a kind gift from Dr W Rainey (Department of Physiology, Medical College

**Table 1** Characteristics of patients and adrenocortical tissues of the EMC and the VUMC cohorts.

	EMC		VUMC	
	ACC <i>n</i> =24	ACA <i>n</i> =14	ACC <i>n</i> =9	ACA <i>n</i> =13
Mean age at diagnosis (years)	50 (range 9–74)	45 (range 26–61)	50 (range 22–73)	55 (range 17–79)
Mean follow-up (months)	42 (range 1–187)	37 (range 1–83)	20 (range 0–68)	30 (range 0–87)
Male (%)	7 (29%)	3 (21%)	2 (22%)	4 (31%)
Mean tumour size cm (s.d.)	13.00 (s.d. 6.9)	3.05 (s.d. 2.0)	16.44 (s.d. 6.2)	3.33 (s.d. 2.1) ( <i>n</i> =12)
Secretion				
Androgens	9 (38%)	0 (0%)	1 (13%)	1 (9%)
Glucocorticoids	13 (54%)	6 (43%)	5 (63%)	3 (27%)
Mineralocorticoids	0 (0%)	5 (36%)	0 (0%)	1 (9%)
Precursors	3 (13%)	0 (0%)	0 (0%)	0 (0%)
Estradiol	4 (17%)	0 (0%)	0 (0%)	0 (0%)
Nonsecreting	8 (33%)	3 (21%)	3 (38%)	6 (55%)
Weiss score (mean)	6.00 (range 3–8)	0.14 (range 0–1)	5.33 (range 3–8)	0.31 (range 0–2)
ENSAT				
I	0 (0%)	12 (86%)	0 (0%)	13 (100%)
II	13 (54%)	2 (14%)	4 (44%)	0 (0%)
III	3 (13%)	0 (0%)	4 (44%)	0 (0%)
IV	8 (33%)	0 (0%)	1 (11%)	0 (0%)
Metastasis	17 (71%)	0 (0%)	6 (67%)	0 (0%)

The two patients with tumours with an unclear diagnosis from the EMC cohort are not included in the table. In the VUMC cohort, one patient with ACC and two patients with ACA did not receive a thorough hormonal workup, so data are missing. EMC, Erasmus University Medical Center; ENSAT, European Network for the Study of Adrenal Tumors; VUMC, VU University Medical Center.

of Georgia, Augusta, GA, USA). Culture conditions were described in detail previously (van Koetsveld et al. 2006). We performed incubations with and without the demethylating drug 5'-Aza-2'-deoxycytidine (AZA), purchased from Sigma-Aldrich. For treatment, we added AZA to the medium for 72 and 168 h. For 168 h of treatment, we refreshed the medium after 72 h and added AZA again. Based on the concentration of AZA that induced 50% cell growth inhibition ( $IC_{50}$ ), we chose three different concentrations of AZA (0.01, 0.05 and 1  $\mu$ M) for the experiments. At the end of the incubation period, we removed medium and collected cells for DNA or total RNA isolation as described below.

### mRNA expression analysis

We isolated total RNA from ACC cells using the High Pure RNA Purification Kit and total RNA from tissues using the High Pure RNA Tissue Kit (both from Roche) according to manufacturer's protocol. To synthesize cDNA, we added 500 ng mRNA template to 40  $\mu$ L Super RT buffer (HT Biotechnology Ltd, Cambridge, UK) containing 40 nmol dNTP, 20 U RNA'sin, 15 ng oligo-dT, 4 U Super RT. After 1-h incubation at 40°C, we diluted the cDNA five times. We mixed 7.5  $\mu$ L TaqMan Universal PCR Master Mix (Applied Biosystems) with concentrations of primers and probes described in Supplementary Table 1, with 5  $\mu$ L cDNA template. Real-time quantitative PCR was performed by TaqMan Gold nuclease assay (Perkin-Elmer) and the ABI-PRISM-7900 Sequence Detection System (Perkin-Elmer), according to manufacturer's protocol. PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT1*) was used to normalize mRNA levels. We calculated PCR efficiencies (E) for the primer-probe combinations (Supplementary Table 1, Sigma-Aldrich) and calculated relative expression of genes using the comparative CT method  $2^{-\Delta\Delta CT}$ .

### Bisulphite conversion and pyrosequencing

We isolated DNA from ACC cells and adrenocortical tissues using the Wizard Genomic DNA Purification Kit (Promega). We treated genomic DNA (1 ng to 1  $\mu$ g) with sodium bisulphite using the EpiTect Plus DNA Bisulfite Kit (Qiagen) following manufacturer's protocol.

Each PCR mix of 50  $\mu$ L contained 0.2  $\mu$ M of forward and reverse primer (one of them being biotinylated), 0.25 mM dNTPs, 2 mM MgCl<sub>2</sub>, 15  $\mu$ L 10 $\times$  PCR buffer, 1.5 U enzyme and 10 ng bisulphite DNA, using the FastStart High Fidelity PCR System kit (Roche). PCR programmes

were as follows: initial denaturation at 95°C for 10 min, 45 cycles of 95°C for 30 s, primer-specific hybrid temperature (Supplementary Table 2) for 30 s, 72°C for 30 s and final extension at 72°C for 10 min. We confirmed amplification of all samples with agarose gel (1%) electrophoresis.

We designed a pyrosequencing assay of previously reported CpGs involved in expression of *IGF2* using Pyromark Assay Design (Supplementary Table 2). After binding of the PCR product to streptavidin-coated Sepharose beads (GE Healthcare), we washed the template, made it single-stranded and neutralized the sample. When the negative control showed a signal during test phase of the primers, we developed new primers in the same region. We performed pyrosequencing using the PyroGold SQA reagent kit (Qiagen) according to manufacturer's protocol and performed analyses on the Pyromark Q24 system. To test reproducibility, we treated five times the same sample of high and low methylated DNA (Epigendx, Hopkinton, MA, USA) with bisulphite and analysed the assays.

### Statistical analysis

We performed statistical analyses by SPSS version 21 and GraphPad Prism 3.0. In all analyses,  $P < 0.05$  was considered significant. Values represent mean  $\pm$  s.d., unless otherwise specified. One-way ANOVA, followed by Tukey's multiple comparison test, was used to test for significant differences in methylation and expression after treatment with AZA. To assess significant differences in methylation per region and *IGF2* mRNA expression in ACCs, ACAs and normal adrenals, we used the nonparametric Mann-Whitney *U* test. For all correlation analyses within the carcinoma group, we used Spearman's correlation.

We quantified the mean methylation in the different regions and *IGF2* expression in the tumour by creating a score for all tumours, defined as the absolute standard deviation score (|SDS|) per region compared with methylation or expression in normal adrenals. We used receiver operating characteristic (ROC) analysis to determine regions with an individually significant predictive value for the diagnosis. We included the three most discriminating CpGs in these regions in final analysis. To assess the explained variation of *IGF2* expression by these factors, we used multiple linear regression. We determined diagnostic accuracy of the |SDS| by performing ROC analysis with mean |SDS| per region for predicting the diagnosis by the pathologist. We performed the same analysis when discriminating only metastatic ACCs versus all other tumours. We determined

the performance of the predictive test by carrying out 10-fold cross-validation. The data were randomly partitioned into 10 subsamples, where after we performed ROC analysis 10 times, in which each subsample was used once as the validation set. An independent cohort was used to validate test performance and the proposed cutoff values. Interobserver variability of the Weiss score was quantified by using an intraclass correlation, while the interrater variability of the binary parameter of pathological diagnosis was quantified by the kappa coefficient.

## Results

### Methylation status of the *IGF2* regulatory regions in adrenocortical tissue

For analysis, we included 24 ACCs, 14 ACAs and 11 normal adrenals. Methylation patterns are shown in [Supplementary Fig. 2](#). A median methylation of 81% was found in DMR0 in ACCs, 77% in ACAs and 82% in normal adrenals ([Fig. 2A](#)). In DMR2, a median methylation of 24% was found in ACCs, 33% in ACAs and 33% in normal adrenals ([Fig. 2B](#)). In both regions, there were no statistically significant differences in median methylation percentages between ACCs and ACAs.

CTCF3 showed a median methylation of 78% in ACCs, 48% in ACAs and 51% in normal adrenals ([Fig. 2C](#)). Methylation in ACCs was significantly higher compared with methylation in ACAs ( $P=0.018$ ). CTCF6 demonstrated a median methylation of 57% in ACCs, 40% in ACAs and 47% in normal tissue ([Fig. 2D](#)), where methylation in ACCs was significantly higher

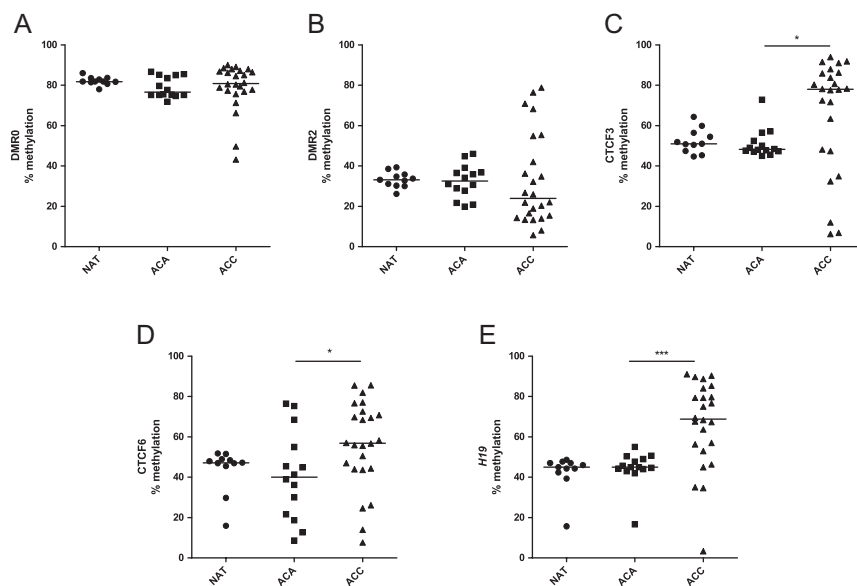
compared with ACAs ( $P=0.034$ ). As expected, the *H19* promoter showed a higher methylation in ACCs as well, namely 69% compared with 45% in ACAs and 45% in normal adrenals ([Fig. 2E](#)). The methylation difference between ACCs and ACAs was also statistically significant ( $P<0.001$ ). For none of the regions there were significant differences in methylation between ACCs and normal adrenals.

### Correlations between DNA methylation in different *IGF2* regulatory regions

The methylation status of CTCF3 and CTCF6, both located in the ICR, were strongly positively correlated in ACCs ( $\rho=0.614$ ,  $P<0.001$ ). Methylation in the *H19* promoter was correlated with methylation in CTCF3, CTCF6 and the DMR0 ( $\rho=0.781$ ,  $P<0.001$ ;  $\rho=0.741$ ,  $P<0.001$ ;  $\rho=0.413$ ,  $P=0.010$ , respectively). Methylation in DMR0 was also correlated with methylation in CTCF3 and CTCF6 ( $\rho=0.595$ ,  $P<0.001$ ;  $\rho=0.361$ ,  $P=0.018$ , respectively), although strongly driven by two low methylated carcinomas. All correlation coefficients and  $P$  values are listed in [Supplementary Table 3](#).

### Expression of *IGF2* mRNA in adrenocortical tissue and correlations with methylation status of different *IGF2* regulatory regions

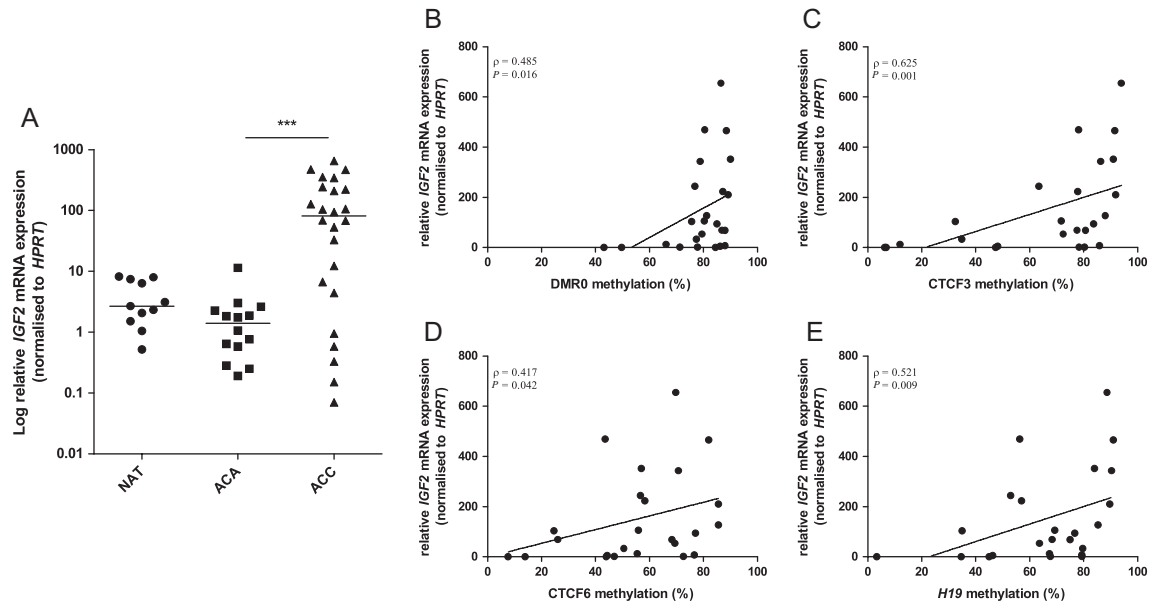
*IGF2* expression was significantly higher in ACCs compared with ACAs ( $P=0.001$ , [Fig. 3A](#)). No statistically significant difference in *IGF2* expression was found between ACAs and normal adrenals.



**Figure 2**

Methylation in the specific regions per group. Every dot represents the mean methylation for every region in a tumour of an individual patient. Lines represent medians. Mean methylations in (A) DMR0, (B) DMR2, (C) CTCF3, (D) CTCF6, and (E) *H19* promoter. \* $P<0.05$ , \*\*\* $P<0.001$ . ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; NAT, normal adrenal tissue.



**Figure 3**

*IGF2* mRNA expression in adrenal tissues and correlation of *IGF2* mRNA expression in ACCs with methylation in *IGF2* regulatory regions. Expression data are presented as scatter dot plot. Expressions are normalized to *HPRT*.  $\rho$  represents Spearman's correlation coefficient. (A) Relative *IGF2* mRNA expression in different groups on a log transformed scale. Lines represent medians. \*\*\**P* < 0.001. (B) Correlation of *IGF2* mRNA expression in ACCs with DMR0 promoter methylation, (C) CTCF3 methylation, (D) CTCF6 methylation, and (E) *H19* methylation. ACA, adrenocortical adenomas; ACC, adrenocortical carcinomas; NAT, normal adrenal tissue.

In ACCs, the mean DMR0, CTCF3, CTCF6 and *H19* promoter methylation were positively correlated with *IGF2* mRNA expression ( $\rho = 0.485$ , *P* = 0.016, Fig. 3B;  $\rho = 0.625$ , *P* = 0.001, Fig. 3C;  $\rho = 0.417$ , *P* = 0.042, Fig. 3D;  $\rho = 0.521$ , *P* = 0.009, Fig. 3E, respectively).

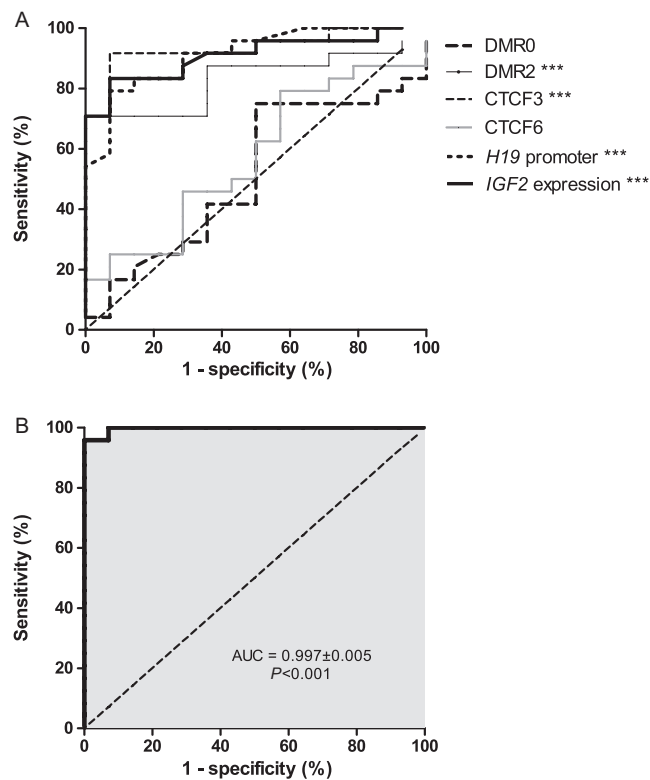
#### Quantification of differences in methylation between carcinomas and adenomas

We calculated SDS per region for the different neoplasms (Supplementary Table 4). *IGF2* expression, DMR2, CTCF3 and the *H19* promoter individually showed a significant predictive value for the diagnosis of ACC (Fig. 4A). When using the mean [SDS] in the second until fourth CpG in DMR2, the fifth until seventh CpG in CTCF3, and the *H19* promoter in a ROC curve for predicting the diagnosis an area under the curve (AUC) of 0.997 was found (Fig. 4B). Test characteristics included sensitivity of 96% and specificity of 100% for a cutoff of [SDS] 2.617, with positive predictive value of 100% and negative predictive value of 93%. These three factors together explained 45.1% ( $R^2$ ) of the variation in *IGF2* expression in ACCs with df 3, F-statistic 5.470 and *P* = 0.007.

Three tumours were difficult to classify based on (only) the Weiss score. Two tumours with a diameter of 9.5 and 6.5 cm had both a Weiss score of 1. However,

the pathologist considered the first tumour as borderline malignant, because it showed minor criteria in the Lin–Weiss–Bisceglia (LWB) system, a modified Weiss system for diagnosis of oncocytic neoplasms (de Krijger & Papathomas 2012). The second case was also difficult to classify, because some criteria, for example, capsular invasion and nuclear polymorphism, were only present in parts of the tumour. Necrosis and a high mitotic rate were not present. When these two tumours with uncertain pathological diagnosis, which were therefore not included in the ACA or ACC group, were tested with the methylation scorings system, they scored [SDS] of 4.29 and 4.20, respectively. This, in turn, would lead in our test to the classification of an ACC. During follow-up (27 and 8 months, respectively), no recurrences or metastases were detected.

A third patient underwent surgery because of a cortisol producing adrenal tumour with the imaging phenotype of an adenoma. Pathological examination showed a Weiss score of 3 with a preferred diagnosis of ACC. However, not all negative prognostic factors (e.g. necrosis, capsular invasion, atypical mitosis) were present in this tumour. Although the diagnosis ACC was not certain, the patient was treated with mitotane as adjuvant therapy. After 20 months, lymph node and subsequently bone metastases developed, confirming the diagnosis of



**Figure 4**

Receiver operating characteristic (ROC) curve of mean absolute standard deviation score (|SDS|) compared with methylation in normal adrenals displayed as sensitivity (y-axis) and 1 – specificity (x-axis) for the diagnosis of adrenocortical carcinoma (ACC) or adrenocortical adenoma (ACA) according to the pathologist. The straight diagonal lines represent reference lines. (A) ROC curve of mean |SDS| compared with methylation in normal adrenals for methylation in all regions and *IGF2* mRNA expression separately. DMR2 ( $P=0.001$ ), CTCF3 ( $P<0.001$ ), the *H19* promoter ( $P<0.001$ ), and *IGF2* expression ( $P<0.001$ ) showed individually significant predictive value for diagnosis of ACC or ACA. (B) ROC curve of the *H19* promoter, second until fourth CpG in the DMR2 and fifth until seventh CpG in CTCF3 for the diagnosis of ACC or ACA according to the pathologist. The area under the curve (AUC) was  $0.997 \pm 0.005$ ,  $P<0.001$ . Cutoff value: mean |SDS| 2.617, sensitivity 96%, specificity 100%.

ACC. The methylation score of this tumour showed a mean |SDS| of 4.68, which indicates malignancy based on our proposed test.

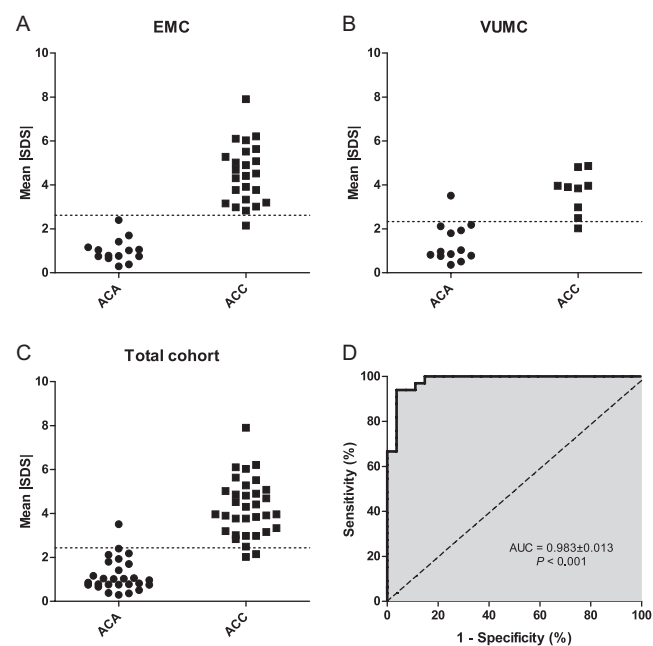
The mean |SDS| of the three regions did not significantly differ between ACCs with and without metastasis at the end of follow-up. When we segregated cases with adverse clinical outcome, that is, metastatic disease, from all other cases, an AUC of  $0.900 \pm 0.049$  ( $P<0.0001$ ) was found.

A 10-fold cross-validation resulted in a good prediction of the diagnosis in 95% of the cases, with a mean cutoff of |SDS|  $2.555 \pm 0.22$ . Mean sensitivity was  $96 \pm 2.0\%$ , specificity was  $100 \pm 0.0\%$  and AUC was  $0.997 \pm 0.002$ . Mean coefficient of variation (CV) of

regions included in final analysis in high methylated samples was 0.039 and in low methylated samples was 0.380. CVs for all regions are described in [Supplementary Table 5](#).

### Validation of the proposed diagnostic test in an independent cohort

After exclusion of four samples due to insufficient DNA yield, 9 ACCs and 13 ACAs were included in the validation cohort, based on the diagnosis determined by the pathologist at the VUMC. There was high agreement between the two pathologists (VUMC and EMC) regarding the Weiss score ( $r=0.914$ ), with one discordant final pathological diagnosis ( $\kappa=0.904$ ). The mean |SDS| was assessed in the three regions proposed on the basis of the EMC cohort ([Fig. 5A](#) and B). ROC analysis, using the diagnosis as determined by



**Figure 5**

Mean absolute standard deviation scores (|SDS|) of the *H19* promoter, second until fourth CpG in the DMR2, and fifth until seventh CpG in CTCF3 in the two different cohorts for adrenocortical adenomas (ACA) and carcinomas (ACC), as determined by the local pathologist on the basis of the Weiss score. Mean |SDS| in the EMC cohort (A), the VUMC cohort (B), and for the total cohort (C). Horizontal dotted lines represent the optimal cutoff value per cohort, as determined by receiver operating characteristic (ROC) curve. (D) ROC of the mean |SDS| for the diagnosis of ACC or ACA according to the pathologist in the total cohort, combining the specimens from the EMC and VUMC. The area under the curve (AUC) was  $0.983 \pm 0.013$  with a sensitivity of 94% and a specificity of 96% using a cutoff of mean |SDS| 2.442 for diagnosing malignancy. The straight diagonal line represents reference line. EMC, Erasmus University Medical Center; VUMC, VU University Medical Center.

the pathologist from the VUMC, showed an AUC of  $0.957 \pm 0.039$  with a sensitivity of 89%, specificity of 92% and a cutoff value of |SDS| 2.331. When we applied the cutoff value obtained from the EMC cohort (2.617) to the validation cohort, a sensitivity of 75% and a specificity of 92% were found. This sensitivity increased to 88% when the pathological diagnosis obtained at the EMC was used. The mean |SDS| of the tumour with discordant pathological diagnosis by the two pathologists was 2.49, and follow-up was only 2 months. Combination of the two cohorts (ACA  $n=27$ , ACC  $n=33$ ) showed an AUC of  $0.983 \pm 0.013$  with a sensitivity of 94% and a specificity of 96% using a cutoff of mean |SDS| 2.442 (Fig. 5C and D).

### Effect of AZA treatment on DNA methylation and expression of *IGF2* and *H19* mRNA in human ACC cell lines

In order to determine the role of DNA methylation on *IGF2* and *H19* expression, we evaluated the effect of the demethylating drug AZA in three human ACC cell lines (H295R, HAC15 and SW13). After 7 days of 1  $\mu$ M AZA treatment, the mean methylation in all regulatory regions in H295R decreased with  $51.9 \pm 2.4\%$ ,  $P < 0.0001$  (mean  $\pm$  s.e.), in a both time-dependent and concentration-dependent manner (Supplementary Fig. 3A, B, C, D, E and H, I, J, K, L). Concomitant with the decrease in methylation, we observed a strong and statistically significant decrease in *IGF2* mRNA expression after 7 days of treatment with AZA in H295R ( $-79.4 \pm 3.8\%$ ,  $P < 0.01$ , mean  $\pm$  s.e.m.), again in a both time-dependent and concentration-dependent manner (Supplementary Fig. 3F and M). The decrease in *IGF2* mRNA expression was accompanied by strongly increased *H19* mRNA expression (Supplementary Fig. G and N). We found similar results for methylation percentages and expression data in HAC15 cells (data not shown). SW13 methylation patterns were partly different compared with H295R and HAC15 and SW13 cells did not express *H19* or *IGF2* mRNA (data not shown).

### Discussion

In the current study, we found that a combined DNA methylation score of three different *IGF2* regulatory regions discriminates ACCs and ACAs with a sensitivity of 96% and specificity of 100%. For tumours of the adrenal cortex, the chance on malignant behaviour increases with

tumour size. The differentiation between ACC and ACA on histopathological grounds can be challenging, by both a poor reproducibility and by tumours classified as being of uncertain malignant behaviour (Pohlink et al. 2004, Tan et al. 2005, Klibanski et al. 2006, Giordano 2010). Mitotane treatment is given to prevent disease recurrence in patients with malignant adrenal tumours but is associated with serious toxicity (Lacroix 2010, Fassnacht et al. 2013). A reliable biomarker that indicates malignant behaviour, particularly in 'borderline malignant' tumours, could be helpful for the decision on postoperative strategies, like the choice on adjuvant mitotane and the duration and intensity of follow-up.

If we would have used this test for the patient with a Weiss score of 3 who developed metastasis after 20 months, the methylation score could have been supportive for the decision of adjuvant mitotane treatment. For the two cases originally classified as tumours with 'uncertain behaviour', but are identified as ACC according to our proposed test, close monitoring is currently performed. Further follow-up, which is now 27 and 8 months from diagnosis, will have to elucidate whether these tumours will recur or metastasize and whether the methylation score indeed offers benefit compared with the Weiss score in these cases. At present, no recurrences or metastases developed in both patients.

In this study, we confirmed that *IGF2* is an important marker for ACC, but as also previously described in a number of studies, does not fully discriminate ACCs from ACAs (Creemers et al. 2016). Mean sensitivity and specificity for diagnosing malignancy are both around 80%, with substantial variances between studies. *H19* seems to be an important regulator, given the positive correlation of the *H19* promoter methylation with *IGF2* expression in our study, as well as in previous research (Gao et al. 2002). By pyrosequencing of different regions in a tumour of the same patient, we were able to define the most discriminating regions in this dataset and propose a diagnostic test with very high accuracy. Choosing a smaller subset of CpGs provided even better test characteristics.

We used three different adrenocortical cancer cell lines to demonstrate the mechanism of regulation of *IGF2*. By treating adrenocortical cancer cells with the demethylating agent AZA, we were able to strongly decrease the *IGF2* mRNA expression and increase the *H19* mRNA expression. Herewith, we confirmed DNA methylation as a strong regulatory element in *IGF2* and *H19* expression. Although it is still unclear whether *IGF2* is mainly a contributor or an effect of



malignancy (Drelon *et al.* 2012, Guillaud-Bataille *et al.* 2014), further research should be done in order to find new insights in therapeutic possibilities of demethylating drugs, considering the reversibility of IGF2 mRNA expression.

Pyrosequencing is a technology yielding accurate and highly reproducible knowledge about methylation percentages at a CpG (Tost & Gut 2007, Quillien *et al.* 2012). By analysing normal adrenals and neoplasms, we could quantify to which extent methylation in tumours deviated from normal. The methylation patterns in DMR2 and CTCF3 visually revealed three phenotypes in ACCs: hypomethylated, moderately methylated and hypermethylated (Fig. 2F and I, respectively). The concept of IGF2 regulation is based on instability of the IGF2/H19 locus. By transforming methylation in absolute SDS, we identified both hypomethylated and hypermethylated phenotypes. Additionally, methylation of DMRs can have various effects on gene expression, both silencing and activation (Moore *et al.* 1997). Methylation of CpGs in promoter regions generally cause repression of the gene (Bell & Beck 2010).

It is important to take into account heterogeneity of adrenocortical tumours, since this can provide different percentages of methylation in different parts of the tumour.

A notable consideration in this study is the reference diagnosis, considering the only definite diagnosis of ACC after metastasis. We have set the diagnosis of the tumours as conclusive as possible, taking into account histopathological features and presence of metastasis (71% of all ACCs). The AUC of  $0.900 \pm 0.049$  for discriminating metastatic ACCs vs all other tumours requires the consideration that there are ACCs reported with no adverse outcome and long survival (>10 years), in literature as well as in our cohort.

The aberrant methylation patterns in ACCs can be explained either by loss of heterozygosity or by *de novo* (de)methylation. Probably in most ACCs, aberrant methylation patterns are caused by deletion of the maternal allele, a structural abnormality mostly seen in malignant adrenal tumours (Gicquel *et al.* 1997). However, this cannot be the case in all ACCs based on the methylation patterns. It is important to notice that the underlying cause of these methylation patterns will not influence the test characteristics.

This diagnostic test, based on analysis in only DNA, has an advantage for both reproducibility and time consumption. Besides, it makes the test more practical

and useful, since RNA is more instable and fragile. We do need to take into account that our analyses are performed on frozen specimens, which is at the moment not readily available in all centres. Future research could focus on the possibility of assessing methylation percentages on DNA obtained from formalin-fixed paraffin-embedded specimens, improving the diagnostic applicability of our proposed test.

Our findings were validated in an independent cohort, which largely increases the generalizability of the proposed diagnostic test. However, larger studies, requiring multicentre collaborations, due to the rare incidence of ACCs, will be necessary to fully validate the predictive value of these specific epigenetic changes and to also validate the required cutoff values for malignancy. These results should be compared with urine steroid metabolomics, a recently introduced new potential preoperative marker for malignancy in adrenal tumours (Arlt *et al.* 2011).

In conclusion, we show that specific methylation patterns of IGF2 regulatory regions are a promising tool to assess malignancy in adrenal tumours and could lead to the first objective diagnostic tool for identifying ACCs.

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0266>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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