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Epigenetic dysregulation in adrenocortical carcinoma, a systematic review of the literature

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ABSTRACT

Adrenocortical carcinoma (ACC) is a rare and aggressive endocrine malignancy with a poor prognosis. Diagnosis and treatment of this tumor remains challenging. The Weiss score, the current gold standard for the histopathological diagnosis of ACC, lacks diagnostic accuracy of borderline tumors (Weiss score 2 or 3) and is subject to inter observer variability. Furthermore, adjuvant and palliative systemic therapy have limited effect and no proven overall survival benefit. A better insight in the molecular background of ACC might identify markers that improve diagnostic accuracy, predict treatment response or even provide novel therapeutic targets. This systematic review of the literature aims to provide an overview of alterations in DNA methylation, histone modifications and their potential clinical relevance in ACC.

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1. Background

Adrenocortical carcinoma (ACC) is an endocrine malignancy with a prevalence of 0.7–2 per million inhabitants (Fassnacht et al., 2011). The overall prognosis of ACC is poor with reported 5-year overall survival rates ranging between 16% and 44% (Søreide

et al., 1992; Abiven et al., 2006; Luton et al., 1990; Pommier and Brennan, 1992; Icard et al., 2001; Haak et al., 1994; Bellantone et al., 1997). At initial presentation, 48% of the tumors is localized within the adrenal gland (stadium I-II), 16–27% has locoregional invasion or lymph node metastasis (stadium III) and 11–36% of the patients present with distant metastasis (stage IV a-c) (Fassnacht et al., 2009; Libé et al., 2015; Bilimoria et al., 2008). Five-year survival of patients with ACC is stage dependent. From stage I to IV it declines from 82%, towards 61%, 50% and less than 13% respectively (Fassnacht et al., 2011). Surgical resection of locoregional disease

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remains the sole curative treatment option for ACC (Fassnacht et al., 2011). Despite novel insights in the carcinogenesis of this rare and aggressive tumor, accurate diagnosis of borderline adrenocortical tumors remains difficult. More importantly, there is no proven effective adjuvant therapy for ACC that improves overall survival.

Accurate diagnosis of a subcategory of “borderline” adrenocortical tumors with a Weiss score of 2 or 3 may be challenging (Giordano, 2010; de Krijger and Papathomas, 2012; Lau and Weiss, 2009). The Weiss score is accepted as the gold standard for histopathological diagnosis of adrenocortical tumors due to its accuracy, simplicity and reliability (Weiss et al., 1989; Aubert et al., 2002). However, the score has a low accuracy for accurate diagnosis of borderline adrenocortical tumors (de Krijger and Papathomas, 2012; Lau and Weiss, 2009). Some patients initially diagnosed with benign borderline tumors develop local recurrence during follow-up, sometimes with distant metastasis (Giordano, 2010; Klibanski et al., 2006; Pohlink et al., 2004). Current understanding about carcinogenesis of borderline tumors is hampered because of a low number of reported cases, limited follow-up and heterogeneity of endpoints (de Krijger and Papathomas, 2012). The identification of molecular diagnostic markers that differentiate ACCs from adrenocortical adenoma (ACA) might be beneficial for the diagnostic accuracy of this subcategory of adrenocortical tumors (Giordano, 2010).

Currently, the only FDA approved adjuvant treatment of ACC is the adrenocorticolytic mitotane (Lysodren®). Adjuvant treatment with mitotane may increase recurrence-free survival but does not affect overall survival (Terzolo et al., 2007; Postlewait et al., 2016). On top of this, treatment with mitotane comes with substantial, mainly gastrointestinal and neurological side effects. (Hoff and Berruti, 2016). According to consensus agreements and guidelines, adjuvant treatment with mitotane is indicated for patients with locoregional, high risk disease following surgical resection or for patients with metastatic disease (Berruti et al., 2010; Kulke et al., 2015). The Ki67 index is one of the main parameters used for patient selection for adjuvant therapy. However, its predictive capacity for treatment response has not been assessed. Furthermore, the scoring of Ki67 in tumor cells is subject to inter observer variability, which greatly affects the estimated survival (Papathomas et al., 2016). The identification of additional molecular markers may improve the selection of patients that could benefit from adjuvant treatment.

Over the last decades, multiple molecular alterations have emerged as factors in ACC carcinogenesis.

Whole genome gene expression analysis of ACC identified two transcriptomic clusters with distinct gene expression patterns and survival. These clusters, denominated as C1a and C1b, corresponded with a five-year overall survival of 20% and 91% respectively (Assié et al., 2010; de Reyniès et al., 2009). Altered gene expression levels can be caused by many factors, both genetic and non-genetic. Mutations and copy number alterations may be one of these factors and are frequently reported in ACC. Mutations in genes involved in Wnt/ β -catenin signaling such as *CTNNB1*, *ZNRF3*, *APC* and *MEN1* can be found in up to 40% of ACCs, while the p53-Rb pathway is the second most affected signaling route (33% of ACCs) with alterations like inactivating mutations or homozygous deletions of *TP53* (Assié et al., 2014). The proportion of *TP53* mutations in benign adrenocortical tissues is low, whereas β -catenin mutations can be found in both ACCs and ACAs (Ohgaki et al., 1993; Reincke et al., 1994; Libé et al., 2007; Tissier et al., 2005).

Epigenetic mechanisms are another factor involved in the regulation of gene expression. DNA methylation is one of these epigenetic regulatory mechanisms. In normal cells, DNA methylation is responsible for selective regulation of gene expression. The

process of DNA methylation is mediated by DNA methyl transferases (DNMTs) and may occur throughout the genome in C-phosphate-G (CpG) dinucleotides. The genome is characterized by an overall low CpG content, except for areas in gene promoter regions. The CpG dinucleotides cluster in gene promoter regions, so called CpG islands (Takai and Jones, 2002). Methylation of CpG islands in these regions mechanically blocks transcription by creating a physical barrier (Rodriguez et al., 2010). Methyl-CpG binding proteins (MBD proteins) adhere to hypermethylated DNA regions and induce chromatin remodeling by recruiting histone deacetylases (HDACs) and chromatin remodeling complexes (Ballestar and Wolffe, 2001; Prokhortchouk and Hendrich, 2002). The opposite occurs in hypomethylated CpG areas. Methylation of CpG islands may affect gene expression, for example by silencing oncoregulatory genes. As such, an increased prevalence of CpG island methylation has been proposed to be involved in carcinogenesis of several tumors. The exact molecular basis of this phenomenon remains to be explored (Hughes et al., 2013). Denominated as CpG-island methylation phenotype (CIMP), this methylation pattern was identified for the first time in colon carcinoma (Toyota et al., 1999). Subsequently, CIMP was identified in multiple other malignancies (Liu et al., 2008; Strathdee et al., 2001; Ueki et al., 2000; Arai et al., 2012; Abe et al., 2005; Tanemura et al., 2009). Although some similarities exist between the CIMP patterns in different tumors, it is likely that the molecular basis of the CIMP phenotype is tumor specific. Nevertheless an exact definition of (tumor specific) CIMP phenotypes is lacking (Hughes et al., 2013).

A second epigenetic regulatory mechanism involved in the regulation of chromatin and gene expression are histone modifications. Histones form the spool on which double stranded DNA is wound to form nucleosomes. A nucleosome consists of 146 base-pairs wrapped around 8 histones. The eight histones are formed from 2 copies of H2A, H2b, H3 and H4. The amino acids forming the histone core and tails can be modified by various mechanisms such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination and ADP-ribosylation (Bannister and Kouzarides, 2011). These mechanisms may affect the order of amino acids, ultimately forming a “histone code” which can attract enzymes that in turn could influence the chromatin structure. Acetylation and deacetylation of histone tails is performed by histone modifying proteins such as histone acetyltransferases (HATs) and HDACs (Bird and Wolffe, 1999; Jones and Baylin, 2002). Ultimately, the level of chromatin condensation influences gene expression. If relaxed due to a mechanism such as hypomethylation, chromatin allows for transcription and gene expression is promoted. Hypermethylation may cause chromatin to condense, thereby blocking transcriptional factors and silencing gene expression (Verdone et al., 2006; Kuo and Allis, 1998; de Ruijter et al., 2003). Epigenetic changes are defined as heritable chromosomal modifications that affect gene expression without changing the coding DNA sequence itself. These alterations might attribute to carcinogenesis (Kulis and Esteller, 2010; Clark and Melki, 2002; Mukamel and Tanay, 2013; Paz et al., 2003). Epigenetic dysregulation may affect gene expression either directly by regulating gene transcription or indirectly by affecting translation through factors such as non-coding RNAs. The role of non-coding RNAs in ACC has been extensively reviewed previously (Hassan et al., 2017; Igaz et al., 2015). Therefore, this review will focus on DNA methylation, histone modifications and their potential clinical relevance in ACC. These epigenetic mechanisms may help to differentiate ACC from ACA. Furthermore, alterations of these mechanisms in ACC might help in the selection of patients that can benefit from adjuvant therapy.

2. Methods

2.1. Search strategy

Data collection and analysis were performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2015). The Intelligent Gateway to Biomedical & Pharmacological Information (EMBASE) and the National Library of Medicine (MEDLINE/PubMed) were systematically searched. The search strategy is provided in [supplementary table 1](#). All records published until 28th February 2017 were searched. Abstracts were independently screened by 2 authors (PJ & VM) and non-relevant articles were excluded based on in- and exclusion criteria. References of articles included for full-text review were checked for relevant publications. Full-text review of remaining records was performed by both authors (PJ & VM) independently. Non-relevant articles were excluded according to in- and exclusion criteria. In case of disagreement, consensus was reached through discussion with all authors.

2.2. Selection of studies

Inclusion criteria were English written studies reporting on methylation or histone modification in ACC in humans. Reviews, conference abstracts, case reports, non-English articles, in vitro studies, animal studies and studies in patients <18 year were excluded.

3. Results

The initial search of EMBASE and MEDLINE identified 376 records, of which 127 were duplicates. Abstract analysis excluded 221 records. One additional record was identified via references of records included for full-text review. Following full text-review of 30 articles, 6 articles were removed based on in- and exclusion criteria. Finally, 24 articles were included for this review ([Fig. 1](#), [supplementary table 2](#)). This systematic literature review aimed to provide an extensive overview of all relevant articles regarding DNA methylation and histone modifications in adult patients with ACC. The applied search strategy aimed to maximize sensitivity and not specificity for articles regarding these subjects. The downside of the method applied for this review lies in the fact that studies were not assessed for quality of their study design. The majority of studies included for this review focused on alterations in DNA methylation and their potential relevance for future clinical applications. Dysregulated DNA methylation patterns might be highly relevant for the differentiation of ACCs from benign adrenal tissues or to predict overall survival for individual patients.

3.1. Genome wide DNA methylation patterns and their association with survival

Six studies applied whole genome methylation profiling to characterize adrenocortical tumors based on methylation levels (Assié et al., 2014; Rechache et al., 2012; Barreau et al., 2013; Zheng et al., 2016; Legendre et al., 2016; Gara et al., 2015). When relevant, we will describe the methods used for analysis of methylation data and hierarchical clustering to illustrate possible biases that may explain inter study variability in reported results. Each of these studies quantified methylation levels using either β -value or M-value metrics. The β -value has a more intuitive biological representation. It is preferable to use the M-value for methylation analysis due to its superior statistical validity, expressed by a better detection rate (DR) and true positive rate (TPR) for both highly methylated and unmethylated CpG sites (Du et al., 2010).

Three methylation profiling studies compared methylation levels between ACC, ACA and normal adrenocortical tissue (NAC) (Rechache et al., 2012; Legendre et al., 2016; Gara et al., 2015). The first whole genome methylation profiling study identified a global hypomethylation signature (adjusted $P \leq 0.01$) by comparing 8 ACC, 12 metastatic ACC, 48 ACAs and 19 NAC samples (Rechache et al., 2012). Furthermore, it was found that hierarchical clustering (unknown metric) of methylation profiling data could separate ACC from benign adrenal tissue (ACA and NAC) with the exception of one ACC and one benign adrenal sample that consistently clustered with samples from the opposite histotype (Rechache et al., 2012). Rechache analyzed whole genome methylation levels of CpG islands, shores (0–2 kb from CpG islands), shelves (2–4 kb from CpG islands) and open sea loci (isolated loci without destination) using the Infinium Human-Methylation (HM)450 Beadchip (Illumina Inc, San Diego, CA). This assay includes 485,000 CpG methylation sites, covering 99% of RefSeq genes, 96% of CpG islands and 92% of CpG shores. Methylation data was quantified with the β -value method (cutoff values $P \leq 0.01$ and $\Delta\beta \leq -0.20$ or $\Delta\beta \geq 0.20$). Legendre quantified the difference of methylation levels between 17 ACCs, 1 metastatic ACC and 6 NAC samples. This study confirmed a significant ($P < 0.0001$) level of hypomethylation in ACC compared to normal adrenocortical tissue (cutoff values $P \leq 0.01$ and $\Delta\beta \leq -0.20$ or $\Delta\beta \geq 0.20$) (Legendre et al., 2016). Furthermore, it was found that unsupervised cluster analysis (Euclidian distance) could separate ACCs from NACs based on altered methylation levels (Legendre et al., 2016). The third study that applied the HM450 array to assess methylation levels between ACC and normal tissue analyzed 10 ACCs, 75 ACAs and 2 NACs (Gara et al., 2015). It was seen that methylation differences between ACC and NAC were higher compared to ACC and ACA. However, no statistical information was provided to illustrate these findings. 70–77% of the hypomethylated areas that differentiate ACC from ACA and normal adrenocortical tissue are situated in open sea regions, whereas hypermethylated areas are mainly situated in CpG island promoter regions (40–50%) (Rechache et al., 2012; Legendre et al., 2016).

The level of hypermethylation of CpG promoter regions in ACC is inversely correlated with overall survival (Assié et al., 2014; Zheng et al., 2016; Barreau et al., 2011; Jouinot et al., 2016). The first study that described this correlation also proposed to categorize ACCs based on methylation level in CpG-island methylation phenotype (CIMP) groups (Barreau et al., 2013). This study used the Infinium HM27 Beadchip array (Illumina Inc, San Diego, CA) to analyze methylation levels of 51 ACCs and 84 ACAs ([supplementary table 2](#)). The HM27 analyses methylation levels of 27,578 CpG sites in proximal promoter regions of 14,475 genes. Methylation data was quantified using the M-value method, followed by unsupervised hierarchical clustering of the top 2824 CpGs with the highest methylation variability ($SD > 1.5$) in ACC (Manhattan metric, Ward method). First, two distinct methylation clusters were identified. These were denominated as non-CIMP (significantly hypermethylated compared to ACA) and CIMP (significantly hypermethylated compared to ACA and non-CIMP). The CIMP cluster was further subdivided in a CIMP-high (highest methylation level) and CIMP-low cluster (intermediate methylation level). No information about the significance of methylation levels between CIMP-high and CIMP-low clusters was provided, which may suggest that this was not significant. ACCs assigned to a CIMP and non-CIMP cluster were associated with 5-year overall survival of $\pm 35\%$ and $\pm 62\%$ respectively (log rank $P = 0.04$). The prognosis of CIMP-high compared to non-CIMP ACCs was even poorer, with 5-year overall survival of 0% and $\pm 62\%$ respectively ($P = 0.006$). More recently, the hypothesis on the existence of three methylation clusters in ACC (CIMP-high, CIMP-intermediate and CIMP-low) was confirmed by a

Fig. 1. Flowchart of the systematic search of EMBASE and MEDLINE.

study from The Cancer Genome Atlas (TCGA) network (Zheng et al., 2016). The authors also concluded that assignment to each of these methylation clusters is associated with a different overall survival (log-rank p-value 0.0001). The patients in the CIMP-high and CIMP-low clusters had a three-year overall survival of less than 50% or 96% respectively. Methylation levels of 79 ACCs included in the TCGA study were acquired using Illumina HM450 arrays (supplementary table 2) and quantified with β -value metrics and subsequent unsupervised clustering of the most variable 1% of CpG island promoter probes (Euclidean metrics, partitioning around medoids). Although the method used for data analysis is different from Barreau et al. Zheng concluded that the identified methylation clusters in both studies are similar. Interestingly, another study identified four distinct methylation clusters in ACC; CIMP-high, CIMP-low and two clusters within the non-CIMP group (Assié et al., 2014). The first non-CIMP cluster was characterized by hypomethylation of CpG sites outside CpG islands, while the second non-CIMP cluster had low number of differentially methylated CpG sites. The reported 5-year overall survival for CIMP-high, CIMP-low and the two non-CIMP clusters was 0%, $\pm 35\%$, 50% and 90% respectively. The authors applied the same methodology as Barreau et al. to analyze the methylation levels of 51 ACCs and 30 ACAs with the Illumina Infinium HM27 assay. However, they applied a recursively partitioned mixture model (RPM) using the 10% most variant CpG sites for cluster analysis, which is different from methods used by both Barreau and Zheng (Houseman et al., 2008).

We may conclude from these studies that methylation levels can be used to distinguish ACCs from ACAs and normal tissues. For the highest proportions of ACC, the level of methylation of DNA

promoter regions is inversely correlated with overall survival. Furthermore, ACCs can be categorized in clusters with distinct methylation patterns and overall survival. The three key studies that defined methylation clusters in ACC used different methods to assess methylation levels and define robust methylation clusters (Assié et al., 2014; Barreau et al., 2013; Zheng et al., 2016). The reported CIMP-phenotypes therefore might represent categories with different biological behavior. A possible explanation for conflicting results regarding the number of methylation clusters might be the proportion of most variant CpG sites used for cluster analysis. Inclusion of a higher proportion of aberrant methylated CpG sites might provide the statistical power to define a larger number of distinct methylation clusters. Furthermore, a lack of a definition of the ACC “CIMP-phenotype” might have attributed to different methodologies. This may be another explanation why one study identified four CIMP-phenotypes in ACC, while two other studies observed three CIMP. Future studies should define the number of methylation clusters based on consensus. As described below, some studies already validated ACC methylation clusters in separate cohorts. Methylation levels might be useful as tool to differentiate ACCs from ACAs or to predict overall survival. CIMP and non-CIMP phenotypes may help to identify patients with poor prognosis. Prospective studies are warranted to define consensus based methylation clusters and to establish the diagnostic and therapeutic relevance of aberrant methylation levels in ACC. Interestingly, each of key studies uploaded their data in the public domain. This comes with the possibility to combine raw methylation assay data from each study and re-analyze this dataset with a consensus based methodology. Subsequently, a methylation probe set that

accurately describes the predefined CIMP-phenotype can be designed and validated in either FFPE tissue or a prospective cohort. Ultimately, this might attribute to the establishment of a well-defined, reproducible and clinical relevant CIMP-phenotype in ACC (Hughes et al., 2013).

3.2. DNA methylation and gene expression

The identification of CpG islands in promoter regions of individual genes that are specifically hypermethylated in ACC might help to differentiate ACC from ACA or NAC. Since the level of DNA methylation is inversely correlated with overall survival, identification of hypermethylated genes with low mRNA expression might also be relevant to predict prognosis. 16 studies included for this review reported both methylation and mRNA expression levels for individual genes in ACC and ACA and/or NAC (Rechache et al., 2012; Barreau et al., 2013; Zheng et al., 2016; Legendre et al., 2016; Gao et al., 2002; Simi et al., 2010; Korah et al., 2013; Mitsui et al., 2014; Pilon et al., 2015; Creemers et al., 2016a, 2016b; Fonseca et al., 2012; Cheng et al., 2017; Hofland et al., 2014; Liu-Chittenden et al., 2017; Sidhu et al., 2005). Two of these studies found low methylation levels in promoter regions of *TP53* and *MGMT*, hypothesizing that other mechanisms are involved in the regulation of these genes in ACC (Creemers et al., 2016a; Sidhu et al., 2005). The remaining 14 studies described a relation between one or more hypermethylated promoter regions and subsequent downregulated expression of the associated gene (supplementary table 4). Interestingly, a poor overlap exists between hypermethylated genes with low mRNA expression identified in these studies. Only 14 genes were reported by two or more studies (Table 1). A possible explanation for the poor overlap of the reported hypermethylated and downregulated genes between studies could be found in differences in analyzed tissue types, number of samples analyzed and methodologies used for the analysis itself (supplementary table 2). Another cause of the inter-study heterogeneity might be the variation in definitions used for hypermethylated, downregulated genes (supplementary table 3). The *H19* gene was the most frequently reported individual gene that is both hypermethylated and subsequently has low mRNA expression (supplementary table 4). Already in 1994, the first alteration in DNA methylation in ACC was reported at the 11p15 locus, encoding *H19* and *IGF2* (Gicquel et al., 1994). The maternal copy of 11p15 in normal adrenal tissue is unmethylated at the Imprinting Center Region (ICR) 1. This region is associated with expression of the non-coding tumor suppressor *H19* in normal adrenocortical tissue, while the ICR1 region of the paternal copy is methylated and expresses *IGF2*. ACCs have a significant higher methylation levels at *H19* promoter regions compared to ACAs or normal tissue, which causes low *H19* and high *IGF2* mRNA expression levels (Gao et al., 2002; Creemers et al., 2016b; Nielsen et al., 2015). This is likely to be caused by paternal uniparental disomy at the 11p15 locus, a late genetic events in progression of benign adrenal adenomas towards ACC (Nielsen et al., 2015; Gicquel et al., 1997). LOH of the 11p15 locus can be found in nearly 95% of ACCs (Gicquel et al., 1997). Interestingly, some ACCs with similar LOH and tumor phenotypes have normal *IGF2* and moderate *H19* expression levels, which might be caused by a decreased ICR1 methylation levels (Guillaud-Bataille et al., 2014). Altogether, differentially methylated control regions at the 11p15 locus are involved in ACC carcinogenesis and may differentiate ACCs from benign tissues. More recently, mRNA expression of *RARRES2* was found to be downregulated as a consequence of promoter hypermethylation (Rechache et al., 2012; Liu-Chittenden et al., 2017). *RARRES2* overexpression in ACC induced reduced cell invasion, cell proliferation and tumorigenicity in vitro and in vivo.

These observations might be caused by reduced phosphorylation and degradation of β -catenin or phosphorylation of p38 mitogen-activated protein kinase (MAPK). These mechanism were found to be immune-independent, because it does not depend on the recruitment of CMKLR1-expressing immune cells in vitro (Liu-Chittenden et al., 2017). The mechanisms behind hypermethylation and subsequent low mRNA expression in ACC are not reported for the remaining 12 genes (*CD74*, *HSD38B2*, *S100A6*, *CYP7B1*, *GIPC2*, *GYPC*, *GOS2*, *TM7SF2*, *RAB34*, *MLH3*, *SPINT2*, *SLC7A4*). (Rechache et al., 2012; Barreau et al., 2013; Zheng et al., 2016; Legendre et al., 2016).

Three studies aimed to provide insight in the correlation between methylation clusters and transcriptomic clusters in ACC (Assié et al., 2014; Barreau et al., 2013; Zheng et al., 2016). Transcriptomic clusters categorize ACCs based on robust clusters that represent patterns of differentially expressed mRNA levels. The characteristics of a robust cluster are dependent on the methods and cutoff values used to define the cluster. As discussed previously, variation in both methods and cutoff values might lead to different outcomes. This may result in difficulties when comparing results from hierarchical clustering between different studies. Analysis of the methods used to define the transcriptomic clusters of the three aforementioned studies is beyond the scope of this review. With this potential bias in mind, we will describe the relation between methylation transcriptomic clusters as reported by these studies. There is evidence that transcriptomic clusters in ACC are strongly correlated with subgroups based on DNA methylation ($P = 6.3 \times 10^{-5}$, χ^2 test) (Assié et al., 2014). All studies reported data from an integrated analysis of multiple -omic domains, including transcriptomic and methylation data, for individual patients. Each of the three studies identified at least two transcriptomic clusters, consistently denominated as C1a (poor overall survival) and C1b (better overall survival) as described by de Reyniès (de Reyniès et al., 2009). 90–100% of the ACCs with CIMP-high phenotypes identified in these three studies were associated with a C1a transcriptomic phenotype (Assié et al., 2014; Barreau et al., 2013; Zheng et al., 2016). Assié et al. divided ACCs with a C1a phenotype in three subgroups based on their association with DNA methylation clusters. Besides the CIMP-high and CIMP-low phenotypes, a third methylation cluster denominated as “hypomethylated” was associated with the poor prognosis C1a transcriptomic cluster (Assié et al., 2014). Overall, 24–31% of the ACCs with the lowest methylation level phenotypes (denominated as non-CIMP(27,58) or CIMP-low(59)) reported in the three studies were associated with the C1a phenotype. This could be interesting, because the reported 5-year overall of these low methylation level ACC phenotypes ranges between 65 and 96%, while it ranges between 20 and 35% for ACCs associated with the C1a transcriptomic cluster (Assié et al., 2014; Barreau et al., 2013; Zheng et al., 2016). This observation might suggest the existence of a subcategory of hypomethylated ACCs that is associated with a poor prognosis transcriptomic phenotype.

Altogether, 14 genes reported in 2 or more studies have both promoter hypermethylation and lower mRNA expression in ACC compared to benign adrenal tissue. Methylation levels of CpG promoter regions of these genes may be useful to differentiate ACC from ACA or NAC. Furthermore, 24–31% of ACCs with a hypomethylation phenotype (mainly associated with a good overall survival) were associated with a C1a transcriptomic phenotype (associated with a poor prognosis). This observation might affect the power of methylation levels to predict prognosis.

3.3. Histone modifications in ACC

This systematic literature review identified one study that aimed to identify altered mRNA expression levels of genes involved

Table 1
Hypermethylated and downregulated genes in Adrenocortical Carcinoma.

Gene	Location	Function	Reference
<i>CD74</i>	5q32	MHC class II antigen processing	(Rechache et al., 2012; Zheng et al., 2016)
<i>HSD3B2</i>	1p12	Mineralobiosynthesis of all classes of hormonal steroids	(Rechache et al., 2012; Barreau et al., 2013)
<i>S100A6</i>	1q21	Cellular calcium signaling	(Rechache et al., 2012; Zheng et al., 2016)
<i>CYP7B1</i>	8q21.3	Primary bile acids synthesis	(Rechache et al., 2012; Zheng et al., 2016)
<i>GIPC2</i>	1p31.1	Unknown	(Rechache et al., 2012; Zheng et al., 2016)
<i>GYPC</i>	2q14.3	Regulating red cell stability	(Rechache et al., 2012; Barreau et al., 2013)
<i>H19</i>	11p15.5	Non-protein coding tumor suppressor	
<i>G0S2</i>	1q32.2	Promoting apoptosis, preventing BCL2-BAX heterodimer formation	(Barreau et al., 2013; Zheng et al., 2016)
<i>TM7SF2</i>	11q13.1	Lanosterol to cholesterol conversion	(Barreau et al., 2013; Zheng et al., 2016)
<i>RAB34</i>	17q11.2	Protein transport.	(Rechache et al., 2012; Barreau et al., 2013)
<i>MLH3</i>	14q24.3	DNA mismatch repair	(Rechache et al., 2012; Zheng et al., 2016)
<i>SPINT2</i>	19q13.2	Serine protease inhibitor	(Rechache et al., 2012; Legendre et al., 2016)
<i>SLC7A4</i>	22q11.21	Amino-acid transporter	(Rechache et al., 2012; Legendre et al., 2016)
<i>RARRES2</i>	7q36.1	Regulation of adipogenesis, metabolism and inflammation	(Rechache et al., 2012; Liu-Chittenden et al., 2017)

in histone modifications, while another study briefly mentioned altered expression levels of histone modification genes in ACC. The authors of the first study screened mRNA levels of histone methyltransferases, demethylases and associated factors. *EZH2* was identified as the most significantly upregulated histone modifying factor in ACCs. *EZH2* is part of the Polycomb Repressor Complex 2 (PRC2) and functions as catalytic core protein. It catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3), thereby attributing to the silencing of target genes. *EZH2* overexpression is correlated with advanced stages of cancer and poor prognosis (Drelon et al., 2016). High *EZH2* expression levels are related to poor prognosis due to significant expression in the previously described poor prognosis C1A transcriptomic cluster. This may suggest an involvement of this gene in aggressive ACC subtypes. Both gene set enrichment analyses indicated that *EZH2* is associated with proliferation. Zeng briefly reported that mRNA expression levels of histone modification genes (*MLL*, *MLL2*, and *MLL4*) and chromatin remodeling genes (*ATRX* and *DAXX*) were dysregulated in 22% of the analyzed samples (Zheng et al., 2016). Future studies should further clarify the clinical relevancy of the altered mRNA expression of histone modifying genes in ACC.

3.4. The clinical application of epigenetic signatures characterizing ACC

Before the epigenetic signatures that characterize ACC can be clinically applied, development and subsequent validation of tools that accurately quantify these signatures are warranted. This requires a well-defined and validated methylation probe set that either accurately differentiates ACC from ACA or identifies distinct CIMP-phenotypes in ACC.

Multiple studies reported that ACC might be differentiated from benign tissues based on methylation levels (Rechache et al., 2012; Barreau et al., 2013; Legendre et al., 2016; Creemers et al., 2016b). The only study that investigated the accuracy of DNA methylation levels for diagnostic purposes focused on methylation levels of *H19*/*IGF2* regulatory regions (Creemers et al., 2016b). In this study a discovery cohort of 24 ACCs, 14 ACAs and 11 NACs was established to determine the combination of differentially methylated areas that yielded the highest diagnostic accuracy. Mean methylation levels for ACCs and ACAs were quantified as absolute standard deviation scores (|SDS|) compared to NAC methylation levels. The most optimal sensitivity of 96% and specificity of 100% (cutoff value mean |SDS| 2.617) was observed when methylation levels of *H19* promoter region, 2nd – 4th CpG in DMR2 and 5th – 7th CpG in CTCF 3 were used to predict prognosis. A sensitivity of 89% and specificity of 92% (cutoff value mean |SDS| 2617) was calculated

from an independent validation cohort of 9 ACCs and 13 ACAs. Based on DNA methylation levels of the selected regions with the highest diagnostic accuracy, “borderline” tumors excluded for study participation were predicted to be ACCs with an |SDS| of 4.29 and 4.20 respectively. While follow-up is ongoing, it is interesting to note that these patients did not develop metastasis during a follow-up period of 27 and 8 months respectively. Therefore, *H19* promoter methylation status might be helpful to improve diagnostic accuracy of adrenocortical tumors. However, the majority of methylation assays used in clinical practice to diagnose colorectal, prostate or bladder cancer use a combination of two or more molecular markers to obtain an adequate diagnostic power (Imperiale et al., 2014; Church et al., 2014; Stewart et al., 2013; Partin et al., 2014; Renard et al., 2010). It is therefore plausible that additional molecular markers are needed to acquire a sufficient accuracy for a methylation based diagnostic test in ACC that accurately diagnoses borderline tumors.

Zheng aimed to identify a probe set that could accurately predict the CIMP-phenotypes as defined in their study (Zheng et al., 2016). The probe set was established by applying a random forest classifier together with the Boruta method (no cut-off values provided), using the 1% most variable probes commonly expressed from the Illumina Infinium HM450 arrays of two methylation profiling cohorts (Assié et al., 2014; Zheng et al., 2016). The number of ACC samples used for this analysis was not reported. Finally, a set of 68 methylation probes was selected and categorized ACCs to their predefined CIMP phenotypes with 92% accuracy. To validate the prognostic value of these probes, a second cohort of 83 patients was classified in CIMP-phenotypes. Patients assigned to the CIMP-high category were associated with a poor 3-year overall survival of less than 50%, while patients in the CIMP-low category had a three-year overall survival of 96%. These results are similar to the overall survival of matching CIMP phenotypes in the discovery cohort, suggesting that this probe set accurately may predict CIMP phenotypes and prognosis (Zheng et al., 2016).

Barreau et al. reported that multiplex ligation-dependent probe amplification (MS-MLPA) can be used to identify CIMP-phenotypes in ACC and predict overall survival and disease free survival based on methylation levels (Barreau et al., 2013; Jouinot et al., 2016). Based on these observations, a recent study identified 4 methylation probes (*GSTP1*, *PYCARD*, *PAX6* and *PAX5*) as a simplified molecular tool to predict prognosis using the MS-MLPA platform (Jouinot et al., 2016). The probes were selected from a set of 27 methylation probes, based on their correlation with CpG island methylation status as defined by the Illumina Infinium HM27 array (Barreau et al., 2013) (Pearson correlation, $P < 0.05$), association with disease free survival and overall survival (Cox regression,

$P < 0.05$) in a retrospective training cohort of 50 ACCs. Subsequently, the 4 probes were tested in a retrospective validation cohort of 203 ACCs. Methylation levels measured by MS-MLPA were a significant prognostic factor of disease free survival ($P < 0.0001$) and overall survival ($P < 0.0001$). Multivariate analysis showed that the prognostic value of methylation is independent from the gold standard KI67 index and tumor stage for recurrence (HR 1.012, $P = 0.0005$) and death (HR 1.014, $P = 0.0006$) per 1% increase of methylation levels. The authors combined methylation, KI67 index and ENSAT stage in a prognostic score that robustly predicted disease free survival (log-rank $P < 10^{-16}$) and overall survival (log-rank $P < 10^{-16}$).

These first steps towards the translation of altered DNA methylation levels in ACC towards clinical application are promising. The studies indicate that altered DNA methylation patterns might be useful to differentiate ACC from benign adrenal tumors and to predict survival. Future, prospective studies in larger cohorts should be executed to validate these results and quantify the accuracy of pyrosequencing and MS-MLPA for diagnostic and prognostic purposes in ACC.

4. Conclusion

Epigenetic regulatory mechanisms have emerged as a potential clinically relevant factor in ACC. The most important clinical benefits of epigenetic dysregulation in ACC might be found in the improvement of diagnostic tools and prediction of the benefit of individual patients for adjuvant treatment. The main epigenetic markers that could be relevant for these purposes are DNA methylation levels and CIMP-phenotypes. The therapeutic potential of epigenetic alterations in ACC is unclear and needs further research. Future studies should also focus on the feasibility of diagnostic tests that predict diagnosis of adrenocortical tumors based on altered methylation levels. Furthermore, a consensus based CIMP-phenotype needs to be established and requires subsequent prospective validation in independent cohorts.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2017.08.009>.

References

- Abe, M., Ohira, M., Kaneda, A., Yagi, Y., Yamamoto, S., Kitano, Y., et al., 2005 Feb 1. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res.* 65 (3), 828–834.
- Abiven, G., Coste, J., Groussin, L., Anract, P., Tissier, F., Legmann, P., et al., 2006 Jul. Clinical and biological features in the prognosis of adrenocortical cancer: poor outcome of cortisol-secreting tumors in a series of 202 consecutive patients. *J. Clin. Endocrinol. Metabol.* 91 (7), 2650–2655.
- Arai, E., Chiku, S., Mori, T., Gotoh, M., Nakagawa, T., Fujimoto, H., et al., 2012 Aug. Single-CpG-resolution Methylation Analysis Identifies Clinicopathologically Aggressive CpG Island Methylator Phenotype Clear Cell Renal Cell Carcinomas. *Carcinogenesis*. Oxford University Press vol. 33(8):1487–1493.
- Assié, G., Guillaud-Bataille, M., Ragazzon, B., Bertherat, J., Clauser, E., 2010 May. The pathophysiology, diagnosis and prognosis of adrenocortical tumors revisited by transcriptome analyses. *Trends Endocrinol. Metab.* 21 (5), 325–334.
- Assié, G., Letouze, E., Fassnacht, M., Jouinot, A., Luscap, W., Barreau, O., et al., 2014 Jun. Integrated genomic characterization of adrenocortical carcinoma. *Nat. Genet.* 46 (6), 607–612.
- Aubert, S., Wacrenier, A., Leroy, X., Devos, P., Carnaille, B., Proye, C., et al., 2002 Dec. Weiss system revisited: a clinicopathologic and immunohistochemical study of 49 adrenocortical tumors. *Am. J. Surg. Pathol.* 26 (12), 1612–1619.
- Ballestar, E., Wolffe, A.P., 2001 Jan. Methyl-CpG-binding proteins. Targeting specific gene repression. *Eur. J. Biochem.* 268 (1), 1–6.
- Bannister, A.J., Kouzarides, T., 2011 Mar. Regulation of chromatin by histone modifications. *Cell Res. Nat. Publ. Group* 21 (3), 381–395.
- Barreau, O., de Reyniès, A., Wilmot-Roussel, H., Guillaud-Bataille, M., Auzan, C., René-Corail, F., et al., 2011 Nov 23. Clinical and pathophysiological implications of chromosomal alterations in adrenocortical tumors: an integrated genomic approach. *J. Clin. Endocrinol. Metabol.* 93 (2), E301–E311.
- Barreau, O., Assié, G., Wilmot-Roussel, H., Ragazzon, B., Baudry, C., Perlemonne, K., et al., 2013 Jan. Identification of a CpG island methylator phenotype in adrenocortical carcinomas. *J. Clin. Endocrinol. Metabol.* 98 (1), E174–E184.
- Bellantone, R., Ferrante, A., Boscherini, M., Lombardi, C.P., Crucitti, P., Crucitti, F., et al., 1997 Dec. Role of reoperation in recurrence of adrenal cortical carcinoma: results from 188 cases collected in the Italian National Registry for Adrenal Cortical Carcinoma. *Surgery* 122 (6), 1212–1218.
- Berruti, A., Fassnacht, M., Baudin, E., Hammer, G., Haak, H., Lebouilleux, S., et al., 2010 Aug 10. Adjuvant therapy in patients with adrenocortical carcinoma: a position of an international panel. *J. Clin. Oncol.* 28 (23), e401–e402 [author reply e403].
- Bilimoria, K.Y., Shen, W.T., Elaraj, D., Bentrem, D.J., Winchester, D.J., Kebebew, E., et al., 2008 Dec 1. Adrenocortical carcinoma in the United States: treatment utilization and prognostic factors. *Cancer* 113 (11), 3130–3136.
- Bird, A.P., Wolffe, A.P., 1999 Nov 24. Methylation-induced repression—belts, braces, and chromatin. *CELL* 99 (5), 451–454.
- Cheng, J.Y., Brown, T.C., Murtha, T.D., Stenman, A., Juhlin, C.C., Larsson, C., et al., 2017 Mar. A novel FOXO1-mediated dedifferentiation blocking role for DKK3 in adrenocortical carcinogenesis. *BMC Cancer* 17 (1), 164.
- Church, T.R., Wandell, M., Lofton-Day, C., Mongin, S.J., Burger, M., Payne, S.R., et al., 2014 Feb. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut* 63 (2), 317–325.
- Clark, S.J., Melki, J., 2002 Aug 12. DNA methylation and gene silencing in cancer: which is the guilty party? *Oncogene* 21 (35), 5380–5387. Nature Publishing Group.
- Creemers, S.G., Van Koetsveld, P.M., Van Den Dungen, E.S.R., Korpershoek, E., van Kemenade, F.J., Franssen, G.J.H., et al., 2016 Dec. Inhibition of human adrenocortical cancer cell growth by temozolomide in vitro and the role of the MGMT gene. *J. Clin. Endocrinol. Metab.* 101 (12), 4574–4584.
- Creemers, S.G., Van Koetsveld, P.M., van Kemenade, F.J., Papathomas, T.G., Franssen, G.J.H., Dogan, F., et al., 2016 Sep. Methylation of IGF2 regulatory regions to diagnose adrenocortical carcinomas. *Endocr. Relat. Cancer. Biosci.* 23 (9), 727–737.
- de Krijger, R.R., Papathomas, T.G., 2012 Jan. Adrenocortical neoplasia: evolving concepts in tumorigenesis with an emphasis on adrenal cortical carcinoma variants. *Virchows Arch.* 460 (1), 9–18.
- de Reyniès, A., Assié, G., Rickman, D.S., Tissier, F., Groussin, L., René-Corail, F., et al., 2009 Mar 1. Gene expression profiling reveals a new classification of adrenocortical tumors and identifies molecular predictors of malignancy and survival. *J. Clin. Oncol.* 27 (7), 1108–1115. American Society of Clinical Oncology.
- de Ruijter, A.J.M., van Gennip, A.H., Caron, H.N., Kemp, S., van Kuilenburg, A.B.P., 2003 Mar 15. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.* 370 (Pt 3), 737–749.
- Drelon, C., Berthoin, A., Mathieu, M., Ragazzon, B., Kuick, R., Tabbal, H., et al., 2016 Jul. EZH2 is overexpressed in adrenocortical carcinoma and is associated with disease progression. *Hum. Mol. Genet.* 25 (13), 2789–2800.
- Du, P., Zhang, X., Huang, C.-C., Jafari, N., Kibbe, W.A., Hou, L., et al., 2010 Nov 30. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics. Biomed. Cent.* 11 (1), 587.
- Fassnacht, M., Johansson, S., Quinkler, M., Bucsik, P., Willenberg, H.S., Beuschlein, F., et al., 2009 Jan 15. Limited prognostic value of the 2004 International Union against Cancer staging classification for adrenocortical carcinoma: proposal for a Revised TNM Classification. *Cancer* 115 (2), 243–250. Wiley Subscription Services, Inc., A Wiley Company.
- Fassnacht, M., Libé, R., Kroiss, M., Allolio, B., 2011 Jun. Adrenocortical carcinoma: a clinician's update. *Nat. Rev. Endocrinol.* 7 (6), 323–335.
- Fonseca, A.L., Kugelberg, J., Starker, L.F., Scholl, U., Choi, M., Hellman, P., et al., 2012 Oct. Comprehensive DNA methylation analysis of benign and malignant adrenocortical tumors. *Genes Chromosom. Cancer* 51 (10), 949–960.
- Gao, Z.-H., Suppola, S., Liu, J., Heikkilä, P., Jänne, J., Voutilainen, R., 2002 Mar. Association of H19 promoter methylation with the expression of H19 and IGF-II genes in adrenocortical tumors. *J. Clin. Endocrinol. Metabol.* 87 (3), 1170–1176.
- Gara, S.K., Wang, Y., Patel, D., Liu-Chittenden, Y., Jain, M., Boufragech, M., et al., 2015 Oct. Integrated genome-wide analysis of genomic changes and gene regulation in human adrenocortical tissue samples. *Nucleic Acids Res.* 43 (19), 9327–9339.
- Gicquel, C., Bertagna, X., Schneid, H., Francillard-Leblond, M., Luton, J.P., Girard, F., et al., 1994 Jun. Rearrangements at the 11p15 locus and overexpression of insulin-like growth factor-II gene in sporadic adrenocortical tumors. *J. Clin. Endocrinol. Metabol.* 78 (6), 1444–1453.
- Gicquel, C., Raffin-Sanson, M.L., Gaston, V., Bertagna, X., Plouin, P.F., Schlumberger, M., et al., 1997 Aug. Structural and functional abnormalities at 11p15 are associated with the malignant phenotype in sporadic adrenocortical tumors: study on a series of 82 tumors. *J. Clin. Endocrinol. Metabol.* 82 (8), 2559–2565.
- Giordano, T.J., 2010 Oct. Adrenocortical Tumors: an Integrated Clinical, Pathologic, and Molecular Approach at the University of Michigan, 134(10):1440–1443.
- Guillaud-Bataille, M., Ragazzon, B., de Reyniès, A., Chevalier, C., Francillard, I., Barreau, O., et al., 2014. IGF2 promotes growth of adrenocortical carcinoma cells, but its overexpression does not modify phenotypic and molecular features of adrenocortical carcinoma. *PLoS One* 9 (8), e103744.
- Haak, H.R., Hermans, J., van de Velde, C.J., Lentjes, E.G., Gossling, B.M., Fleuren, G.J., et al., 1994 May. Optimal treatment of adrenocortical carcinoma with mitotane:

- results in a consecutive series of 96 patients. *Br. J. Cancer* 69 (5), 947–951. Nature Publishing Group.
- Hassan, N., Zhao, J.T., Sidhu, S.B., 2017 Jan 4. The role of microRNAs in the pathophysiology of adrenal tumors. *Mol. Cell. Endocrinol.* 1–8. Elsevier Ireland Ltd.
- Hoff, A.O., Berruti, A., 2016 Feb. 5th international ACC symposium: future and current therapeutic trials in adrenocortical carcinoma. *Horm. Cancer* 7 (1), 29–35.
- Hofland, J., Steenberg, J., Voorsluijs, J.M., Verbiest, M.M.P.J., de Krijger, R.R., Hofland, L.J., et al., 2014. Inhibin alpha-subunit (INHA) expression in adrenocortical cancer is linked to genetic and epigenetic INHA promoter variation. *PLoS One* 9 (8), e104944.
- Houseman, E.A., Christensen, B.C., Yeh, R.-F., Marsit, C.J., Karagas, M.R., Wrensch, M., et al., 2008 Sep 9. Model-based clustering of DNA methylation array data: a recursive-partitioning algorithm for high-dimensional data arising as a mixture of beta distributions. *BMC Bioinformatics*. Biomed. Cent. 9 (1), 365.
- Hughes, L.A.E., Melotte, V., de Schrijver, J., de Maat, M., Smit, V.T.H.B.M., Bovée, J.V.M.G., et al., 2013 Oct. The CpG island methylator phenotype: what's in a name? *Cancer Res.* 73 (19), 5858–5868.
- Icard, P., Goudet, P., Charpenay, C., Andreassian, B., Carnaille, B., Chapuis, Y., et al., 2001 Jul. Adrenocortical carcinomas: surgical trends and results of a 253-patient series from the French Association of Endocrine Surgeons study group. *World J. Surg.* 25 (7), 891–897.
- Igaz, P., Igaz, I., Nagy, Z., Nyíró, G., Szabó, P.M., Falus, A., et al., 2015 Feb. MicroRNAs in adrenal tumors: relevance for pathogenesis, diagnosis, and therapy. *Cell Mol. Life Sci.* 72 (3), 417–428.
- Imperiale, T.F., Ransohoff, D.F., Itzkowitz, S.H., Levin, T.R., Lavin, P., Lidgard, G.P., et al., 2014 Apr 3. Multitarget stool DNA testing for colorectal-cancer screening. *N. Engl. J. Med.* 370 (14), 1287–1297. Massachusetts Medical Society.
- Jones, P.A., Baylin, S.B., 2002 Jun. The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* 3 (6), 415–428.
- Jouinot, A., Assié, G., Libé, R., Fassnacht, M., Papathomas, T., Barreau, O., et al., 2016 Dec 14. DNA methylation is an independent prognostic marker of survival in adrenocortical cancer. *J. Clin. Endocrinol. Metab.* jc20163205.
- Klibanski, A., Stephen, A.E., Greene, M.F., Blake, M.A., Wu, C.-L., 2006 Nov 23. Case records of the Massachusetts General Hospital. Case 36-2006. A 35-year-old pregnant woman with new hypertension. *N. Engl. J. Med.* 355 (21), 2237–2245.
- Korah, R., Healy, J.M., Kunstman, J.W., Fonseca, A.L., Ameri, A.H., Prasad, M.L., et al., 2013 Aug 5. Epigenetic silencing of RASSF1A deregulates cytoskeleton and promotes malignant behavior of adrenocortical carcinoma. *Mol. Cancer* 12 (1), 87.
- Kulis, M., Esteller, M., 2010. DNA Methylation and Cancer. *Adv Genet* 70, 27–56. Elsevier.
- Kulke, M.H., Shah, M.H., Benson, A.B., Bergsland, E., Berlin, J.D., Blazskowsky, L.S., et al., 2015. Neuroendocrine tumors, version 1.2015. *J. Natl. Compr. Cancer Netw.* JNCN 13, 78–108.
- Kuo, M.H., Allis, C.D., 1998 Aug. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* 20 (8), 615–626.
- Lau, S.K., Weiss, L.M., 2009 Jun. The Weiss system for evaluating adrenocortical neoplasms: 25 years later. *Hum. Pathol.* 40 (6), 757–768.
- Legendre, C.R., Demeure, M.J., Whitsett, T.G., Gooden, G.C., Bussey, K.J., Jung, S., et al., 2016. Pathway implications of aberrant global methylation in adrenocortical cancer. *PLoS One* 11 (3), e0150629.
- Libé, R., Groussin, L., Tissier, F., Elie, C., René-Corail, F., Fratticci, A., et al., 2007 Feb 1. Somatic TP53 mutations are relatively rare among adrenocortical cancers with the frequent 17p13 loss of heterozygosity. *Clin Cancer Res. Am. Assoc. Cancer Res.* 13 (3), 844–850.
- Libé, R., Borget, I., Ronchi, C.L., Zaggia, B., Kroiss, M., Kerkhofs, T., et al., 2015 Oct. Prognostic factors in stage III-IV adrenocortical carcinomas (ACC): an European network for the study of adrenal tumor (ENSAT) study. *Ann. Oncol.* 26 (10), 2119–2125.
- Liu, Z., Zhao, J., Chen, X.-F., Li, W., Liu, R., Lei, Z., et al., 2008 Oct. CpG island methylator phenotype involving tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. *Lung Cancer* 62 (1), 15–22.
- Liu-Chittenden, Y., Jain, M., Gaskins, K., Wang, S., Merino, M.J., Kotian, S., et al., 2017 Jan 23. RARRES2 functions as a tumor suppressor by promoting β -catenin phosphorylation/degradation and inhibiting p38 phosphorylation in adrenocortical carcinoma. *Oncogene* 23, viii6. Nature Publishing Group.
- Luton, J.P., Cerdas, S., Billaud, L., Thomas, G., Guilhaume, B., Bertagna, X., et al., 1990 Apr 26. Clinical features of adrenocortical carcinoma, prognostic factors, and the effect of mitotane therapy. *N. Engl. J. Med.* 322 (17), 1195–1201. Massachusetts Medical Society.
- Mitsui, Y., Yasumoto, H., Nagami, T., Hiraki, M., Arichi, N., Ishikawa, N., et al., 2014 Apr. Extracellular activation of Wnt signaling through epigenetic dysregulation of Wnt inhibitory factor-1 (Wif-1) is associated with pathogenesis of adrenocortical tumor. *Oncotarget* 5 (8), 2198–2207.
- Moher, D., Shamseer, L., Clarke, M., Ghera, D., Liberati, A., Petticrew, M., et al., 2015 Jan 1. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Syst. Rev.* 4 (1), 1.
- Mukamel, Z., Tanay, A., 2013 Jul. Hypomethylation marks enhancers within transposable elements. *Nat. Genet.* 45 (7), 717–718.
- Nielsen, H.M., How-Kit, A., Guerin, C., Castinetti, F., Volland, H.K.M., De Micco, C., et al., 2015 Dec. Copy number variations alter methylation and parallel IGF2 overexpression in adrenal tumors. *Endocr. Relat. Cancer* 22 (6), 953–967.
- Ohgaki, H., Kleihues, P., Heitz, P.U., 1993 May 28. p53 mutations in sporadic adrenocortical tumors. *Int. J. Cancer* 54 (3), 408–410.
- Papathomas, T.G., Pucci, E., Giordano, T.J., Lu, H., Duregon, E., Volante, M., et al., 2016 Apr. An international Ki67 reproducibility study in adrenal cortical carcinoma. *Am. J. Surg. Pathol.* 40 (4), 569–576.
- Partin, A.W., Van Neste, L., Klein, E.A., Marks, L.S., Gee, J.R., Troyer, D.A., et al., 2014 Oct. Clinical validation of an epigenetic assay to predict negative histopathological results in repeat prostate biopsies. *J. Urol.* 192 (4), 1081–1087.
- Paz, M.F., Fraga, M.F., Avila, S., Guo, M., Pollan, M., Herman, J.G., et al., 2003 Mar 1. A systematic profile of DNA methylation in human cancer cell lines. *Cancer Res.* 63 (5), 1114–1121.
- Pilon, C., Rebello, A., Urbanet, R., Guzzardo, V., Cappellesso, R., Sasano, H., et al., 2015. Methylation status of vitamin D receptor gene promoter in benign and malignant adrenal tumors. *Int. J. Endocrinol.* 2015 (1), 375349.
- Pohlink, C., Tannapfe, A., Eichfeld, U., Schmidt, F., Führer, D., Paschke, R., et al., 2004 Jun. Does tumor heterogeneity limit the use of the Weiss criteria in the evaluation of adrenocortical tumors? *J. Endocrinol. Invest.* 27 (6), 565–569.
- Pommier, R.F., Brennan, M.F., 1992 Dec. An eleven-year experience with adrenocortical carcinoma. *Surgery* 112 (6), 963–970 discussion970–1.
- Postlewait, L.M., Ethun, C.G., Tran, T.B., Prescott, J.D., Pawlik, T.M., Wang, T.S., et al., 2016 Apr. Outcomes of adjuvant mitotane after resection of adrenocortical carcinoma: a 13-institution study by the US adrenocortical carcinoma group. *J. Am. Coll. Surg.* 222 (4), 480–490.
- Prokhorchouk, E., Hendrich, B., 2002 Aug 12. Methyl-CpG binding proteins and cancer: are MeCpGs more important than MBDs? *Oncogene* 21 (35), 5394–5399.
- Rechache, N.S., Wang, Y., Stevenson, H.S., Killian, J.K., Edelman, D.C., Merino, M., et al., 2012 Jun. DNA methylation profiling identifies global methylation differences and markers of adrenocortical tumors. *J. Clin. Endocrinol. Metab.* 97 (6), E1004–E1013.
- Reincke, M., Karl, M., Travis, W.H., Mastorakos, G., Allolio, B., Linehan, H.M., et al., 1994 Mar. p53 mutations in human adrenocortical neoplasms: immunohistochemical and molecular studies. *J. Clin. Endocrinol. Metab.* 78 (3), 790–794.
- Renard, I., Joniau, S., van Cleynebreugel, B., Collette, C., Naomé, C., Vlassenbroeck, I., et al., 2010 Jul. Identification and validation of the methylated TWIST1 and NID2 genes through real-time methylation-specific polymerase chain reaction assays for the noninvasive detection of primary bladder cancer in urine samples. *Eur. Urol.* 58 (1), 96–104.
- Rodriguez, C., Borgel, J., Court, F., Cathala, G., Forné, T., Piette, J., 2010 Feb 5. CTCF is a DNA methylation-sensitive positive regulator of the INK/ARF locus. *Biochem. Biophys. Res. Commun.* 392 (2), 129–134.
- Sidhu, S., Martin, E., Gicquel, C., Melki, J., Clark, S.J., Campbell, P., et al., 2005 Jun. Mutation and methylation analysis of TP53 in adrenal carcinogenesis. *Eur. J. Surg. Oncol.* 31 (5), 549–554.
- Simi, L., Malentacchi, F., Luciani, P., Gelmini, S., Deledda, C., Arvia, R., et al., 2010 May. Seladin-1 expression is regulated by promoter methylation in adrenal cancer. *BMC Cancer* 10, 201.
- Sørdeide, J.A., Brabrand, K., Thoresen, S.O., 1992 Jul. Adrenal cortical carcinoma in Norway, 1970–1984. *World J. Surg.* 16 (4), 663–667 discussion668.
- Stewart, G.D., Van Neste, L., Delvenne, P., Delrèe, P., Delga, A., McNeill, S.A., et al., 2013 Mar. Clinical utility of an epigenetic assay to detect occult prostate cancer in histopathologically negative biopsies: results of the MATLOC study. *J. Urol.* 189 (3), 1110–1116.
- Strathdee, G., Appleton, K., Illand, M., Millan, D.W., Sargent, J., Paul, J., et al., 2001 Mar. Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes. *Am. J. Pathol.* 158 (3), 1121–1127.
- Takai, D., Jones, P.A., 2002 Mar 19. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl. Acad. Sci. USA. Natl. Acad. Sci.* 99 (6), 3740–3745.
- Tanemura, A., Terando, A.M., Sim, M.-S., van Hoesel, A.Q., de Maat, M.F.G., Morton, D.L., et al., 2009 Mar 1. CpG island methylator phenotype predicts progression of malignant melanoma. *Clin Cancer Res. Am. Assoc. Cancer Res.* 15 (5), 1801–1807.
- Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P.A., et al., 2007 Jun 7. Adjuvant mitotane treatment for adrenocortical carcinoma. *N. Engl. J. Med.* 356 (23), 2372–2380.
- Tissier, F., Cavard, C., Groussin, L., Perlemonne, K., Fumey, G., Hagnér, A.-M., et al., 2005 Sep 1. Mutations of beta-catenin in adrenocortical tumors: activation of the Wnt signaling pathway is a frequent event in both benign and malignant adrenocortical tumors. *Cancer Res.* 65 (17), 7622–7627.
- Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J.G., Baylin, S.B., Issa, J.P., 1999 Jul 20. CpG island methylator phenotype in colorectal cancer. *Proc. Natl. Acad. Sci. U. S. A.* 96 (15), 8681–8686.
- Ueki, T., Toyota, M., Sohn, T., Yeo, C.J., Issa, J.P., Hruban, R.H., et al., 2000 Apr 1. Hypermethylation of multiple genes in pancreatic adenocarcinoma. *Cancer Res.* 60 (7), 1835–1839.
- Verdone, L., Agrícola, E., Caserta, M., Di Mauro, E., 2006 Sep. Histone acetylation in gene regulation. *Brief. Funct. Genom. Proteomic* 5 (3), 209–221.
- Weiss, L.M., Medeiros, L.J., Vickery, A.L., 1989 Mar. Pathologic features of prognostic significance in adrenocortical carcinoma. *Am. J. Surg. Pathol.* 13 (3), 202–206.
- Zheng, S., Cherniack, A.D., Dewal, N., Moffitt, R.A., Danilova, L., Murray, B.A., et al., 2016 May 9. Comprehensive Pan-Genomic characterization of adrenocortical carcinoma. *Cancer Cell.* 29 (5), 723–736.