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The role of DNA methylation in renal cell carcinoma

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Abstract

Renal cell carcinoma (RCC) is the most common kidney cancer and includes several molecular and histological subtypes with different clinical characteristics. While survival rates are high if RCC is diagnosed when still confined to the kidney and treated definitively, there are no specific diagnostic screening tests available and symptoms are rare in early stages of the disease. Management of advanced RCC has changed significantly with the advent of targeted therapies, yet survival is usually increased by months due to acquired resistance to these therapies. DNA methylation, the covalent addition of a methyl group to a cytosine, is essential for normal development and transcriptional regulation, but becomes altered commonly in cancer. These alterations result in broad transcriptional changes, including in tumor suppressor genes. Because DNA methylation is one of the earliest molecular changes in cancer and is both widespread and stable, its role in cancer biology, including renal cell carcinoma, has been extensively studied. In this review we examine the role of DNA methylation in renal cell carcinoma disease etiology and progression, the preclinical use of DNA methylation alterations as diagnostic, prognostic and predictive biomarkers, and the potential for DNA methylation-directed therapies.

1 Introduction

1.1 Renal cell carcinoma background and challenges

Renal cell carcinoma (RCC) arises from the epithelial cells of the nephron - the functional unit of the kidney. RCC accounts for ~90% of primary malignancies of the kidney and is comprised of several distinct molecular and histological subtypes that have varying clinical characteristics, including therapeutic response [1,2]. While there are over ten recognized subtypes of RCC, the three most common are clear cell RCC (ccRCC, ~75% of cases), papillary RCC (pRCC, ~20% of cases), and chromophobe RCC (chRCC, ~5% of cases). ccRCC also accounts for most kidney cancer-related deaths, typically arises sporadically, and is characterized by cells with clear cytoplasm. pRCC is typically divided into type 1 and type 2 tumors which are distinguished by histopathology differences, while chRCC is usually slower growing with relatively better outcomes [3].

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Over half of all RCCs are diagnosed incidentally during imaging procedures for other conditions, and there are no RCC-specific screening tools available in the clinic [4,5]. When localized, five-year survival rates after surgical resection are high, but over 30% of cases have already spread locally or distantly at the time of diagnosis [6]. When metastatic, RCC is highly resistant to conventional chemotherapy [7,8]. In the past decade, a deepened understanding of the molecular etiology of RCC has led to the development of several effective targeted therapies. These therapies include tyrosine kinase inhibitors that target vascular endothelial growth factor (VEGF) or its receptor, vascular endothelial growth factor receptor (VEGFR) (e.g., axitinib, bevacizumab, pazopanib, sorafenib, and sunitinib), inhibitors of the rapamycin complex 1 (mTORC1) (e.g., everolimus, temsirolimus), immunotherapies (e.g., nivolumab), and multiple kinase inhibitors (e.g., cabozantinib, lenvatinib) [7]. Response rates for these therapies are ~35% and they can increase progression free survival time by months or years, but development of resistance to these therapies is common and most patients still die from their disease [9].

Because of this disease heterogeneity, difficulty in early detection, and inherent resistance to treatment, RCC is one of the ten most common and deadly cancers [6]. In 2017, an estimated 63,990 new cases were diagnosed and 14,400 patients died from the disease in the United States alone [10]. Deletions of the 3p chromosome arm and somatic von Hippel-Lindau (*VHL*) tumor suppressor gene mutations lead to loss of VHL protein expression in a majority of ccRCC cases [11,12]. Likewise, germline mutations of the *VHL* gene produce a highly penetrant inherited syndrome (von Hippel-Lindau disease) in which multifocal ccRCC is a prominent feature. Despite being a driver of development and progression, *VHL* disruption is not sufficient to cause ccRCC in mice [13,14]. Whole genome sequencing studies have indicated mutations in the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex and epigenome modifiers (i.e. BRCA1 associated protein 1, *BAP1*; polybromo1, *PBRM1*; and SET domain containing 2, *SETD2*), are the most frequently mutated genes after *VHL* and contribute to disease pathology by disrupting normal epigenetic programs and altering the tumor genome landscape through widespread gene expression alterations [15,16]. In fact, chromatin remodeling pathway gene mutations were detected in 69.3% of ccRCC, 53.0% of pRCC, and 14.9% of chRCC tissues in The Cancer Genome Atlas (TCGA) data set, demonstrating common molecular pathways disrupted across RCC subtypes [17]. Because it is the most common subtype and accounts for the most patient deaths, the majority of kidney cancer research focuses on ccRCC. A recent preclinical study in mice implicates a multi-step process for ccRCC pathogenesis, first requiring loss of VHL, followed by further inactivation of hypoxia inducible factor 1 alpha subunit (HIF1)/signal transducer and activator of transcription 3 (STAT3) through loss of PBRM1, and finally mTORC1 activation [18].

1.2 DNA methylation and its role in cancer

The covalent addition of a methyl group to a cytosine base results in 5-methylcytosine, altering the chemical properties of the DNA region without changing the sequence. This DNA methylation has an important role in the regulation of the expression of nearby genes and is a key player in many developmental processes including lineage specification and organogenesis, as well as dysregulation in cancer and aging [19–23]. While DNA

methylation changes can be observed throughout the genome, DNA methylation occurring at cytosine and guanine (CpG) dinucleotides is the most studied thus far. These CpGs are not distributed evenly in the genome, but are clustered in dense, CpG-rich regions known as CpG islands, and these are found commonly in gene promoters and highly repetitive sequence regions [24]. Methylated CpG islands aid in maintaining chromosomal stability and typically are associated with suppression of transcription of downstream regions (e.g., methylation in highly repetitive regions silences non-coding DNA and transposable elements). Non-methylated CpG-islands are commonly enriched in the promoter regions of expressed genes. However, the distribution and density of DNA methylation often becomes altered in malignant transformation [25] and cancer cells commonly display decreased DNA methylation in repetitive regions (i.e. hypomethylation) and regional hypermethylation of CpG islands in malignant compared to normal tissues [22]. These changes in methylation can result in significant changes in gene expression that include the activation of oncogenes and silencing of tumor suppressor genes [24]. Because DNA methylation is one of the earliest observable molecular changes in cancer, is stable, and can be quantitatively measured, it is promising for both understanding cancer etiology and progression, as well as identifying clinical biomarkers for disease diagnosis, prognosis, and directed therapies [26,27].

This review summarizes current evidence for the role of DNA methylation in the malignant transformation of kidney cells and discusses the utility and strides made in identifying and applying DNA methylation biomarkers to the detection, diagnosis, prognosis, and therapeutic potentials in kidney cancer, with a particular focus on ccRCC. Papers indexed in PubMed [28] with the keywords 'renal cell carcinoma' and 'DNA methylation' were considered for inclusion.

2 The role of DNA methylation in RCC

2.1 Silencing of tumor suppressor genes

Hypermethylation of CpG islands that silences expression of tumor suppressor genes has been studied in ccRCC for more than 20 years [29]. Early reports demonstrated that the *VHL* promoter is hypermethylated in 11% of RCC tumors and might represent a mechanism for *VHL* silencing independent of gene mutation, accounting for gene and protein expression decreases in those patients without a copy number event or deleterious mutation in *VHL* [30]. More recently, TCGA confirmed these findings by demonstrating 7% of ccRCC cases showed epigenetic silencing at *VHL* [15]. In addition, a recent meta-analysis found that *VHL* promoter hypermethylation was significantly higher in RCC patients compared to non-malignant kidney tissues (odds ratio, OR=7.93, p<0.001), but there was not a significant association between *VHL* methylation and detection methodologies, or clinicopathological characteristics like tumor stage, grade, or size, lymph node status, or histological type [31]. This underscores observations that *VHL* inactivation through promoter methylation (or mutation and deletion) often occurs during, and contributes to, RCC carcinogenesis yet is not sufficient to drive malignant progression.

Since deletions at 3p often encompass the entire chromosomal arm, DNA methylation of genes in that chromosomal segment have been investigated as possible contributors to

ccRCC genesis and progression. One such gene, the Ras association domain family 1 isoform A (*RASSF1A*) has been implicated as a tumor suppressor gene involved in the development of several cancers [32]. *RASSF1A* promoter methylation was described in 32 of 138 primary ccRCC tumors and 12 of 27 primary papillary RCC tumors (the second most common histologic subtype of RCC). *RASSF1A* promoter hypermethylation was associated with absent gene expression, and yet there was no association between *RASSF1A* and *VHL* inactivation [33]. Additionally, two meta analyses identified significant association between *RASSF1A* hypermethylation and RCC risk (OR=19.35, 95% confidence intervals, CI=9.57–39.13; and OR=4.14, 95% CI=1.06–16.1), as well as between *RASSF1A* promoter methylation and tumor grade (OR=3.32, 95% CI=1.55–7.12) [34,35]. Early *ex vivo* and *in vitro* studies also show that DNA methylation may be involved in metastasis to bone through TIMP metalloproteinase inhibitor 2 (TIMP2) regulation [36]. While many other tumor suppressor genes have been shown to undergo epigenetic silencing in RCC [37], it remains to be seen which may be driving cancer etiology and progression and which are passenger effects of genome-wide remodeling.

2.2 Insights from genome-wide DNA methylation profiling

The first genome-wide DNA methylation studies in RCC [27,38,39] identified differential DNA methylation between adjacent benign and malignant kidney tissue with increased methylation associated with more aggressive cancers [12,40,41]. Additional RCC studies have implicated DNA methylation as a potential risk factor or as playing a role in malignant transformation. For example, a retrospective case-control study of almost 900 kidney cancer cases identified decreased global DNA methylation levels, defined based on percentage of 5-methyldeoxycytosine per total levels of deoxycytidine, in patient peripheral blood compared to healthy matched controls [42]. However, other studies have identified regional hypermethylation events, rather than global hypomethylation as most indicative of RCC [43]. Likewise, entropy metrics of DNA methylation have demonstrated that there is greater randomness in lower order methylation of genes associated with cancer (i.e. oncogenes and tumor suppressor genes) in cancer genomes compared with normal kidney [43]. Kidney cancer tissues have also been shown to have widespread DNA hypermethylation in gene bodies and kidney-specific enhancer regions, and that these alterations are linked to dysregulated hypoxia signaling pathways and RCC-cell sensitivity to decitabine, a hypomethylating chemotherapeutic that acts by inhibiting DNA methyltransferase [44].

2.3 Mechanism of DNA methylation alterations

The underlying mechanisms of altered DNA methylation in RCC are unknown, particularly the causes of de novo hypermethylation in discrete regions of the genome. Increased RNA and protein expression of the DNA methyltransferase 3 beta splice variant 4 (DNMT3B4) has been observed in RCC tumors compared to adjacent benign tissue. Since this *DNMT3B* splice variant has been shown to be involved in de novo DNA methylation during development, DNMT3B4 is a strong candidate for causing the DNA methylation changes observed in RCC and other cancers [45]. Additionally, oxidative stress can induce epigenetic reprogramming, including DNA hypermethylation, and thereby drive malignant transformation, and regional hypoxia and differences in activation of hypoxia signaling pathways in RCC can influence the oxidative state of kidney cancer cells [46]. However,

how these hypermethylation events are directed to discrete CpG islands in the genome is unknown.

2.4 Histone methyltransferases and global DNA methylation changes

TCGA analysis of ccRCC identified an association between *SETD2* (a histone methyltransferase) mutations and global DNA hypomethylation outside of gene promoter regions [15], supporting the premise that histone H3K36 trimethylation might help maintain heterochromatin [47]. Furthermore, in RCC cell lines, *SETD2* loss-of-function mutations disrupt both the epigenome and transcriptome through redistribution of histone H3K36 trimethylation, resulting in genome-wide DNA hypermethylation. Thus, through DNA mutation *SETD2* disruption could drive genome-wide changes that result in de-differentiation and cancer progression [48]. However, a recent report in 155 ccRCC patients suggests that low *SETD2* protein expression, and not H3K36me3 expression, is associated with more aggressive cancer [49]. Future research examining both histone methyltransferase activity and other functions of *SETD2* are warranted. Interestingly, truncating *PBRM1* mutations, a very common event in ccRCC that also affects chromatin structure, may also drive DNA methylation events and cancer progression [50]. A recent report suggests both gene expression and DNA methylation changes associated with mutations in chromatin modifier genes are present in clear cell and papillary RCC TCGA tumors and are predictive of worse outcome. These molecular profile changes were also present in a subset of RCC cases without mutations in SWI/SNF complex components, suggesting alterations to chromatin modifier-regulated genes is commonly dysregulated and by multiple mechanisms [2].

2.5 Regulation of microRNAs

DNA methylation has also been implicated in the etiology and progression of kidney cancer though its role in regulating microRNAs, which are small non-coding RNAs that control expression of a targeted set of genes. For example, DNA methylation was shown to lead to transcriptional silencing of the microRNA miR-10b in 9 ccRCC patient tumors and 5 RCC cell lines, and miR-10b has been shown to act functionally as a tumor suppressor [51]. TCGA also reported DNA methylation silencing of miR-10b expression, as well as silencing of miR-21 and miR-30a [15]. DNA methylation silencing of both miR-9-1 and miR-9-3 ($p < 0.001$) has been reported in 74 ccRCC tumor samples compared to paired normal tissues from 32 of these cases and with reduced recurrence-free survival ($p = 0.034$ and $p = 0.007$, respectively) [52].

3 DNA methylation alterations as RCC biomarkers

3.1 Diagnostic biomarkers

RCC survival rates are correlated with tumor stage, and when cancer is confined to the kidney the standard of care is resection. After resection, 5-year survival rates are high, exceeding 90% at 5 years for stage 1 cancers, and underscoring the importance of early detection. Almost half of all renal tumors are identified incidentally [4,5], typically at the time of cross-sectional imaging performed for other reasons, and an increasing number of lesions are very small. Between 20 and 30% of small renal lesions are benign, depending on

the size of the lesion, and this poses a significant challenge in management given the risks and high cost of surgical resection [53]. New methods for distinguishing benign from malignant lesions that are accurate and inexpensive could substantially improve patient care. If accurate, these methods could be used for screening of individuals at high risk for RCC, such as those with germline VHL mutations or Lynch syndrome [54]. RCC DNA methylation changes are a promising source of candidate diagnostic biomarkers because they are found early during carcinogenesis [26], including in precancerous lesions [27]. Additionally, as stable DNA marks that can be quantitatively measured, DNA methylation changes lend themselves to readily available detection strategies [55].

Several studies of RCC have demonstrated the potential for identification of targeted DNA methylation diagnostic biomarkers in patient tumor tissue, urine, and serum. Most of the candidate biomarkers display an increase in DNA methylation in the cancer compared to normal for the practical reason that it is far easier to detect events uniquely changed in cancer (e.g. mutation, hypermethylation) than it is to detect loss of a signal, since lost signals could also occur when there is a technical failure of an assay. Several groups have used methylation specific PCR approaches to detect candidate promoter methylation markers of RCC in the urine of relatively small sets of patients and controls. For example, methylated DNA was detected in at least one of six loci in preoperative urine sediment in 88% of 50 RCC patients compared to none of the normal controls (specificity 100%) [56]. Using a different, although somewhat overlapping set of 9 loci, Hoque et al. detected methylation in at least 1 locus in 23 of 26 (88%) of urine samples and 12 of 18 serum samples (67%), although low level methylation was detected in a few of the normal control urine and serum samples [57]. A study using methyl-sensitive restriction enzymes and PCR for 8 gene promoters in serum from 35 RCC patients, demonstrated that at least one gene was hypermethylated in each patient and that combining multiple genes increased diagnostic sensitivity and specificity to 62.9% and 87%, respectively [58]. However, after enthusiastic initial reports, no validation studies of these specific marker sets have been published.

More recently, detection of methylated DNA sequences has been explored in plasma cell free DNA (cfDNA). A recent study investigated promoter methylation in cfDNA from 157 patients with RCC and 43 patients with benign renal tumors for *VHL*, *RASSF1A*, *RNF185*, *PTGS2*, and *PI6*. Detection of CpG island methylation of *VHL* (area under the receiver operating characteristic curve, AUC ROC = 0.705) and *RASSF1A* (AUC ROC=0.694) were discriminative of RCC from benign disease [59]. More recently, a separate study analyzed plasma samples from 27 RCC patients compared to controls for 6 loci and could detect hypermethylation in over half of RCC patients for 4 of those loci [60]. However, in contrast to the previous study, they could not detect hypermethylation in the VHL promoter region. The reasons for this discrepancy are unclear and could have to do with the sensitivity of the PCR-based assays, particularly since bisulfite treatment adversely affects the DNA template. Given that VHL is hypermethylated infrequently, it is unlikely to serve as a sensitive biomarker for detection [15].

While these studies show promise in developing methylated DNA sequences as biomarkers for RCC detection, one of the challenges is that available biomarkers have poor performance characteristics at baseline, given the infrequency with which they are methylated in RCC

tissues. To identify candidate diagnostic biomarkers, we have carried out a more comprehensive assessment of DNA methylation changes in 96 RCC patients with diverse histologies of RCC. We identified a panel of 27 CpGs that could most reliably distinguish benign adjacent tissue from a pooled sample set that included most of the common RCC histological subtypes. This diagnostic model showed excellent performance characteristics in the discovery set (AUC ROC=0.990) and validated in pooled TCGA data (n>1000 samples) that included clear cell, papillary, and chromophobe carcinomas compared to normal renal tissue (AUC ROC=0.972) [61]. A recent study has validated our approach by identifying a small set of diagnostic markers using TCGA data, and testing its prediction of cancer in 272 ex vivo kidney biopsies, correctly classifying 92% of tumors, 86% of benign tissues, and 98% of adjacent normal tissues [62].

While methylation can be used as a diagnostic tool to distinguish normal from malignant renal lesions, it could also help with classification of histological subtypes of RCC. Unsupervised clustering of DNA methylation profiles can clearly distinguish histological subtypes of RCC [63], as well as proximal- versus distal-tubule-derived kidney tumors [64]. Given that subtypes of RCC have differing responses to therapy and outcomes, DNA methylation profiling could aid in clinical decision-making.

3.2 Prognostic biomarkers

Prediction of prognosis or tumor aggressiveness has become highly relevant to growing populations of patients with RCC, such as older patients with small tumors detected incidentally who are managed with surveillance [65], or patients undergoing surgical resection in whom adjuvant therapy is considered [66]. A remarkable number of methylation biomarkers have been proposed for prognostication of RCC [24,67]. Single loci candidate methylation biomarkers of prognosis include *RCVRN* [68], *CRHBP* [69], *AR* [70], *BMP-2* [71], *CDO1* [72], *DAB2IP* [73], and *KEAPI* [74]. While promising, many of these biomarker studies have design shortcomings, including relatively small sample sizes and lack of validation in independent cohorts [75]. Panels of prognostic biomarkers have also been described, including *GREM1*, *LADI*, *NEFH*, and *NEURL*, and methylation at each site alone or in combination was predictive of patient prognosis in three independent patient cohorts (HR=3.64 and 95% CI=1.02–13.00, HR=7.54 and 95% CI=2.68–21.19, and HR=3.60 and 95% CI=2.02–6.40) [76].

Global genome-wide promoter DNA methylation profiling has also revealed that an increase in the total number of hypermethylated genes was associated with higher tumor grade ($p<0.001$) and worse overall prognosis ($p=0.005$) [77]. A CpG island methylator phenotype (CIMP), defined by an increase in DNA methylation globally across CpG islands, has been identified in a subset of RCCs. In a set of 100 ccRCC patients, 14 tumors were identified as CIMP positive, and these tumors showed worse outcome compared to CIMP negative tumors (HR: 75.8, 95% CI=7.81–735) [78]. Because defects in the spindle checkpoint pathway have been associated with CIMP in ccRCC tumors, this subset of patients may benefit from spindle checkpoint-targeting therapies [79]. Additionally, the second most diagnosed RCC subtype, papillary RCC (pRCC), also has been shown to have a subset of CIMP tumors. These patients had a worse overall survival ($p=1\times 10^{-16}$), accompanied by low

fumarate hydratase (*FH*) gene expression levels [80]. Recently, these patients were also shown to have early-onset disease [17]. *FH* disruption diminishes TET enzyme function, which can in turn disrupt genome-wide DNA methylation [81].

Combined analysis of the clear cell, papillary, and chromophobe RCC TCGA cohorts also identified a subset of tumors with more aggressive disease associated with increased DNA methylation profiles [2]. Increased hypermethylation in all three of these RCC histologic subtypes was associated with decreased patient survival ($p < 0.0001$) [17]. When considering only probes unmethylated in normal kidney tissues, the authors found a set of 1,532 variably hypermethylated probes identifying 240 RCC tumors (10 CIMP RCCs, 182 ccRCCs, 23 type 2 pRCCs, 16 chRCCs, and 9 type 1 and unclassified pRCCs) characterized by increased DNA hypermethylation and worse patient survival ($p < 0.0001$). The authors also identified a subset of metabolically divergent chRCC samples with increased ribose metabolism gene expression and decreased Krebs cycle, 5' AMP-activated protein kinase (*AMPK*), and electron transport gene expression also associated with very poor survival and hypermethylation.

While most studies have focused on promoter DNA methylation, examining DNA methylation patterns outside of the promoter region has demonstrated that many kidney-specific enhancers (regions associated with H3K4Me1 marks), as well as sites enriched for anti-AP2 alpha antibody (AP2a), aryl hydrocarbon receptor precursor (AHR), aryl hydrocarbon receptor nuclear translocator (ARNT), HAIRY, and HIF1 transcription factor binding, are also hypermethylated and associated with patient prognosis [44]. Interestingly, profiling both 5-methylcytosine and 5-hydroxymethylcytosine (oxidized 5-methylcytosine) in patient tumors and RCC cell lines suggests that gene body hypermethylation may actually be linked to loss of 5-hydroxymethylcytosine through *IDH1* down regulation, and that loss is both prognostic ($p < 0.0001$) and a marker of DNA methylation pattern remodeling [82].

The availability of multi-omics datasets in RCC has allowed association of DNA methylation with gene expression patterns to identify relevant pathways activated in RCC and test them for their relationship with patient outcomes. In the TCGA dataset, the strongest inverse correlation between DNA methylation and gene expression was observed for ubiquinol-cytochrome C reductase hinge protein (*UQCRH*), where 36% of tumors showed hypermethylation and methylation increased with higher grade and stage. Up-regulation of the PI3K pathway is common in RCC and leads to a glycolytic shift in the cells, referred to as the Warburg effect. The PI3K pathway is regulated by miR-21 and GRB10, and hypomethylation of miR-21 and hypermethylation of *GRB10* increase PI3K signaling and are associated with worse outcomes [15]. Pathway analysis of TCGA data using DNA methylation and gene expression, has identified four discrete DNA methylation signatures (*PARVG*, *PLCB2*, *RAC2*, and *VAV1*) that are correlated with RCC prognosis ($p = 0.0125$) [83]. Interestingly, a cross-omics screening study found that protein expression and DNA methylation improved prognostic predictions when combined with clinical variables, while gene expression, microRNA expression, and copy number variation did not [84]. Recent progress in methylation profiling in formalin fixed, paraffin embedded (FFPE) tissues will likely expand the number of biomarkers tested, and dramatically expand the number of validation datasets available for biomarker development [85]. However, despite

many reported preclinical biomarkers of diagnosis and prognosis, clinical application has remained elusive [86].

3.3. Predictive biomarkers

As mentioned above, the management of advanced RCC has changed dramatically with the advent of targeted therapies, but identification of patients for whom particular targeted therapies will be effective and which are futile remains a continuing clinical challenge. Several DNA methylation biomarkers predictive of response or lack of response to therapies have been proposed. For example, in the VEG102616 trial, *VHL* status, including *VHL* methylation, did not correlate with response to pazopanib, a first-line VEGF-targeted agent [87]. However, analysis of the DNA methylation status of 48 selected genes from 14 matched fresh frozen RCC tissues before and after treatment with sunitinib (a receptor tyrosine kinase inhibitor) found that *VHL* hypermethylation was the only locus significantly altered after treatment [88]. Hypermethylation of neurofilament heavy (*NEFH*), cystatin 6 (*CST6*), and laminin 1 (*LADI*) have been associated with poor prognosis and poor response to several antiangiogenic therapies in advanced RCC [89,90]. Others have demonstrated the ability to monitor regulatory T cells in peripheral blood lymphocytes by quantitatively measuring DNA methylation of forkhead box transcription factor, *FOXP3*, a specific marker of regulatory T cell development [91]. With this method, a significant increase in regulatory T cells was detected in 18 metastatic RCC patients compared to 12 controls following two immunotherapy cycles, with significantly higher expanded regulatory T cells in non-responders compared to therapy-responding patients [92]. Clearly, additional work is necessary to test the role and predictive value of methylation of specific loci in modulating the response to targeted therapies.

4. DNA methylation and therapy

Given that DNA methylation changes are early, widespread, and consistent events in RCC, an intriguing strategy for treatment is by genome-wide targeting of chromatin regulators and DNA methylation (Table I). In particular, 5-aza-2'-deoxycytidine (also known as decitabine and trade name Dacogen), that inhibits DNA methyltransferase transferase(s) (DNMT) and causes loss of DNA methylation, has shown activity in pre-clinical studies [93]. Altering DNA methylation with 5-aza-2'-deoxycytidine can synergize with other drugs or reverse drug resistance in RCC. For example, 5-aza-2'-deoxycytidine increases the susceptibility of RCC cells to paclitaxel (a tubulin-targeting drug) in vitro, putatively by activating the PI3K/Akt-LEF1/ β -catenin pathway [94,95]. Similarly, organic cation transporter 2 (*OCT2*) is commonly suppressed in RCC through promoter hypermethylation, and treatment with 5-aza-2'-deoxycytidine induces demethylation and re-expression of *OCT2* which sensitizes RCC cells and xenografts to oxaliplatin, a platinum-based antineoplastic [96]. To date, there have been few clinical trials in RCC with DNMT inhibitors. A phase I trial showed that low-dose decitabine could be administered safely along with high-dose cytokine interleukin-2 (IL-2) and that the 3 RCC patients in the trial showed stable disease with therapy [97]. A slightly larger phase II trial that included 14 RCC patients found no measurable antitumor activity in any carcinomas examined, including RCC [98].

Data from methylation profiling studies could provide insights into novel therapeutic strategies in RCC. For example, gene expression profiling of RCC cell lines showed that 5-aza-2'-deoxycytidine suppresses cell growth through G2/M cell cycle arrest and the p38-NF- κ B signaling pathway, suggesting this pathway could be exploited therapeutically [99]. Clear cell RCC tumors with Notch pathway activation due to DNA methylation and copy number variation alterations appear to be clinically distinct and show better overall survival, highlighting the possibility of targeting Notch signaling with or without DNMT inhibitors [103]. Recent studies have also highlighted that 16% of RCC cases have loss of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) through mutation, deletion, or promoter hypermethylation and that this tumor suppressor and cell cycle regulator may be targetable by cyclin-dependent kinase (CDK) 4 and 6 inhibitors [17,104].

Recently, there has been considerable interest in potential synergies between DNA methylation-targeted therapies and immunotherapy. DNMT inhibitors cause upregulation of MHC I levels and increase levels of antigens displayed by MHC I [100]. In addition, treatment with 5-aza-2'-deoxycytidine can induce expression of the cancer testis antigens (CTA) *MAGE-1*, *-2*, *-3*, and *-4*, *GAGE1-6*, and *NY-ESO-1* for which there are targeted immunotherapies [101]. Interferon response gene expression, and in turn interferon-induced apoptosis, has been demonstrated after treating RCC cell lines with 5-aza-2'-deoxycytidine [102]. Many studies in other cancer types suggest that treatment with low-dose hypomethylating agents such as 5-aza-2'-deoxycytidine could prime tumors to respond to immunotherapies, possibly by inducing expression of neo-antigens [100]. Given the success of immunotherapy for treating advanced RCC [105], the possibility of improving responses through modulation of DNA methylation is an exciting future area of preclinical and clinical investigation. Of particular interest, a recent exome sequencing study of 35 metastatic ccRCC patients found that patients with inactivating mutations in *PBRM1* were more responsive to anti-PD-1 monotherapy ($p=0.012$), as were ccRCC patients in a validation cohort ($p=0.0071$, $n=63$). Further gene expression analysis in both cell lines and RCC tumors demonstrated that *PBRM1* loss might alter response to immune checkpoint inhibitor therapy through Janus kinase-signal transducers and activators of transcription (JAK-STAT), immune signaling, and hypoxia pathway expression changes [106].

5. Conclusions and perspectives

Widespread and reproducible methylation changes demonstrate an important role for methylation alterations in renal cell carcinoma. However, much work needs to be done to identify which of the methylation changes are causative in RCC development and progression, and which changes are passengers. Integration of methylation with other genome wide alterations and improvement in pathway annotation and analysis strategies are likely to provide insights into the important methylation changes in RCC. In addition, understanding of the relationship between DNA methylation changes, chromatin states and environmental influences, including exogenous modulators (cadmium, smoking and environmental carcinogens) and local and endogenous factors (tumor microenvironment) will provide insights into the causes of RCC [94].

Growing evidence suggests that DNA methylation changes are a promising source of disease biomarkers that could be used for diagnosis, prognosis, therapeutic monitoring or prediction of response to therapies, perhaps even across RCC subtypes. While population-based screening for RCC is currently not feasible, screening of high risk populations, including those with ambiguous cystic lesions (Bosniak 2 & 3), those with small poorly characterized renal masses, and those with familial syndromes that predispose to RCC could benefit tremendously from a DNA methylation-based test that could be done in the blood or urine, thereby reducing the need for serial radiographic testing with its attendant risks [4,107]. However, considerable challenges remain in using DNA methylation for biomarker development. The extent of tumor heterogeneity with respect to methylation is understudied in RCC. Heterogeneity will affect performance of nearly all classes of biomarkers - most prominently, prognostic and predictive biomarkers, since subclones are likely responsible for progression and resistance to therapy [108]. In addition, development of assays that can reproducibly measure DNA methylation changes has proven to be especially challenging, particularly on low-template samples such as cfDNA in plasma, or in small numbers of cells isolated from the urine or blood (i.e. circulating tumor cells or CTCs). Most methods employ bisulfite treatment conversion of methylated DNA [109], which damages template DNA and does not distinguish between 5- methylcytosine and 5-hydroxymethylcytosine, complicating assay development. Finally, methylation changes can be tissue specific, developmentally regulated, and can change with age [110]. Identification of DNA methylation changes that are specific to RCC, and not found in normal tissue and do not change with age could prove difficult. Further confounding biomarker development, RCC is comprised of diverse histologies with distinct molecular changes that likely extend to DNA methylation. While we have described methylation changes common to the major histologic subtypes [61], these findings require further validation, and minor subtypes with distinct changes could be missed when methylation biomarker tests are applied to patients with an uncharacterized renal mass, thereby eroding the performance characteristics of the test.

Despite promising pre-clinical studies in many cancers, few DNA methylation inhibitors have advanced to clinical application. There are several challenges in developing methylation modulators for therapeutic use including lack of specificity for cancer DNA, significant toxicities, and poor efficacy. Furthermore, the widespread demethylation incurred by available agents can lead to genomic instability and may induce side effects that are as of yet, not fully characterized [111]. However, less toxic DNA demethylating agents are being developed [112,113]. In addition, promising work documenting the role of DNA methylation in modulating drug resistance could lead to novel combination approaches that render cytotoxic and/or targeted therapies effective in previously refractory cancers. Finally, the promise of immunotherapy broadly, and checkpoint inhibitors specifically, for effectively treating RCC opens many opportunities for altering methylation to allow for re-expression of tumor specific antigens and improving responses.

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Key Points

- DNA methylation is an early, widespread, and stable molecular change occurring in cancer.
- This review examines the role of DNA methylation in renal cell carcinoma including in disease etiology and progression, as predictive markers, and with directed therapies.

Table I.

Strategies for RCC treatment targeting chromatin regulators and DNA methylation.

Therapeutic Strategy	Support and/or Mechanism	Studies
5-aza-2'-deoxycytidine(decitabine, Dacogen)	-inhibits DNMT(s), causes loss of DNA methylation -suppresses cell growth through G2/M cell cycle arrest and the p38-NF- κ B signaling pathway -cause upregulation of MHC I levels and increase levels of antigens displayed by MHC I -can induce expression of the cancer testis antigens (CTA) MAGE-1, -2, -3, and -4, GAGE1-6, and NY-ESO-1 for which there are targeted immunotherapies -interferon response gene expression, and in turn interferon-induced apoptosis, has been demonstrated after treatment -could prime tumors to respond to immunotherapies, possibly by inducing expression of neo-antigens	[93,99-102]
5-aza-2'- deoxycytidine + paclitaxel	-5-aza-2'-deoxycytidine increases the susceptibility of RCC cells to paclitaxel (a tubulin-targeting drug) in vitro, putatively by activating the PI3K/Akt-LEF1/ β -catenin pathway	[94,95]
5-aza-2'- deoxycytidine + oxaliplatin	-organic cation transporter 2 (<i>OCT2</i>) is commonly suppressed in RCC through promoter hypermethylation, and treatment with 5-aza-2'-deoxycytidine induces demethylation and re-expression of <i>OCT2</i> which sensitizes RCC cells and xenografts to oxaliplatin, a platinum-based antineoplastic	[96]
Low dose decitabine + high-dose cytokine interleukin-2(IL-2)	-phase I trial demonstrated safety; 3 RCC patients enrolled showed stable disease with therapy -phase II trial; 14 RCC patients found no measureable antitumor activity in any carcinomas examined, including RCC	[97,98]
Notch pathway activation	-ccRCC tumors with Notch pathway activation due to DNA methylation and copy number variation alterations appear to be clinically distinct and show better overall survival, highlighting the possibility of targeting Notch signaling with or without DNMT inhibitors	[103]
CDK4/6 inhibitors	-16% of RCC cases have loss of cyclin-dependent kinase inhibitor 2A (<i>CDKN2A</i>) through mutation, deletion, or promoter hypermethylation and that this tumor suppressor and cell cycle regulator may be targetable by CDK4/6 inhibitors	[17,104]
Anti-PD-1 monotherapy	-patients with inactivating mutations in <i>PBRM1</i> were more responsive to anti-PD-1 monotherapy -gene expression analysis in both cell lines and RCC tumors demonstrated that <i>PBRM1</i> loss might alter response to immune checkpoint inhibitor therapy through Janus kinase-signal transducers and activators of transcription (JAK-STAT), immune signaling, and hypoxia pathway expression changes	[106]