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The role of epigenetics in kidney malignancies

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Introduction Renal cell carcinomas (RCC) are collectively the third most common type of genitourinary neoplasms, surpassed only by prostate and bladder cancer. Cure rates for renal cell carcinoma are related to tumor grade and stage; therefore, diagnostic methods for early detection and new therapeutic modalities are of paramount importance. Epigenetics can be defined as inherited modifications in gene expression that are not encoded in the DNA sequence itself. Epigenetics may play an important role in the pursuit of early diagnosis, accurate prognostication and identification of new therapeutic targets.

Material and methods We used PubMed to conduct a comprehensive search of the English medical literature using search terms including epigenetics, DNA methylation, histone modification, microRNA regulation (miRNA) and RCC. In this review, we discuss the potential application of epigenetics in the diagnosis, prognosis and treatment of kidney cancer.

Results During the last decade, many different types of epigenetic alterations of DNA have been found to be associated with malignant renal tumors. This has led to the research of the diagnostic and prognostic implications of these changes in renal malignancies as well as to the development of novel drugs to target these changes, with the aim of achieving a survival benefit.

Conclusions Epigenetics has become a promising field in cancer research. The potential to achieve early detection and accurate prognostication in kidney cancer might be feasible through the application of epigenetics. The possibility to reverse these epigenetic changes with new therapeutic agents motivates researchers to continue pursuing better treatment options for kidney cancer and other malignancies.

Key Words: kidney cancer ‹› DNA methylation ‹› histone modification ‹› microRNA regulation

INTRODUCTION

Renal cell carcinomas (RCC) represent 2-3% of all non-cutaneous malignant neoplasms in adults of both genders [1]. RCC is not a single entity, but encompasses a group of histologically distinct tumors including clear cell RCC (ccRCC), papillary RCC (pRCC) and chromophobe RCC (cRCC) [2]. The World Health Organization Classification of Renal Tumors established in 2004 recognizes clear cell, papillary, chromophobe, collecting duct and unclassified RCC as the main subtypes of renal tumors [3]. Since clinical outcomes are closely related to tumor stage, diagnostic methods that help to achieve early detection as well as new therapeutic modalities are critical.

Cancers and epigenetics are closely associated, with deoxyribonucleic acid (DNA) hypermethylation being widely accepted as a feature of many cancers [4]. Alterations in DNA methylation have been described in human cancer for more than thirty years now and its importance in cancer research has increased over the last decade [5]. Additional epigenetic mechanisms including histone modifications and MicroRNA (miRNA) regulation have also been reported. In this review, we discuss the role of epigenetics in the prognosis, diagnosis and treatment of kidney malignancies.

Epigenetics

Epigenetics can be defined as inherited modifications in gene expression that are not encoded in the DNA

sequence itself [6]. Epigenetic mechanisms include DNA methylation, histone modifications and miRNA regulation. Aberrant DNA methylation usually occurs in cytosine, guanine (CpG) rich regions and is associated with gene silencing. Histone lysine acetylation at the N-terminal leads to gene activation, whereas histone lysine methylation causes transcriptional activation or repression depending on the position of the methylated lysine rest [7]. De-regulation of miRNA expression (a class of small non-coding RNA) resulting from epigenetic modifications occurring in transformed cells may lead to tumorigenesis [8]. Previous studies were able to demonstrate that the detection of DNA hypermethylation allows for normal tissue to be distinguished from malignant renal tissue. This may allow diagnostic conclusions to be drawn [9]. Some examples of tumor suppressor genes affected by epigenetic changes are listed in table 1.

DNA methylation

DNA methylation consists of an addition of a methyl group (CH3) to the carbon 5 position of the cytosine ring forming a covalent bond. Although most cytosine methylation takes place in the CpG dinucleotide sequence, dinucleotide sequences containing adenine and thymine (CpA, CpT) can also be involved [10]. DNA methylation alterations are one of the most consistent epigenetic modifications occurring during carcinogenesis involving various organ sites [11].

Table 1. *Tumor suppressor genes and associated renal tumor histological type [44-47]*

Tumor Suppressor Gene	Histological type
PBRM1	ccRCC
SMARCB1	RT
SETD2	ccRCC
VHL	ccRCC
APAF1	ccRCC
RASSF1A	ccRCC
APC	ccRCC
E-cadherin	ccRCC
$p14^{ARF}$	ccRCC
$p16^{INK4\alpha}$	ccRCC

PBRM1 – protein polybromo-1 gene; ccRCC – clear cell Renal Cell carcinoma; SMARCB1 – SWI/SNF – related matrix-associated actin-dependant regulator of chromatin subfamily B member 1 gene; RT – Rhabdoid Tumor; SETD2 – SET domain containing 2 gene; VHL – Von Hippel-Lindau gene; APAF-1 – Apoptosis Protease Activating Factor-1; RASSF1A – Ras association domain family 1 isoform A; APC – Adenomatous polyposis coli; $p14^{ARF}$ – p14 (alternate reading frame)

DNA hypermethylation

Hypermethylation inactivates transcription of CpG dinucleotides in promoter regions of tumor suppressor genes leading to gene silencing [12]. Earlier studies have shown that aberrant DNA hypermethylation is involved in the pathogenesis of RCC. One study demonstrated a 100% correlation between DNA hypermethylation of the promoter gene *RASSF1A* and papillary RCC (pRCC) [13]. The important role of promoter hypermethylation with subsequent transcriptional silencing of tumor suppressor genes in the development of RCC was noted by Ricketts et al. In their report, they found that in patients with clear cell RCC (ccRCC), the *von Hippel-Lindau (VHL)* tumor suppressor gene is inactivated by promoter hypermethylation in 15% of cases [14]. Several enzymes called DNA methyltransferases (DNMT) are required to accomplish the process of hypermethylation [15] (Figure1). Hypermethylation may be analyzed by using the sensitive methylation-specific PCR (MSP) technique, which allows for the identification of a single methylated allele

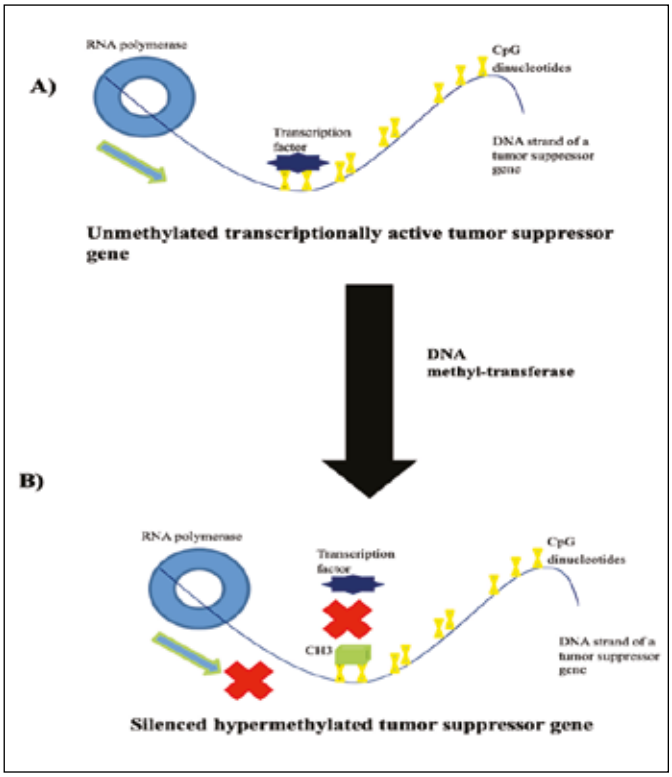

Figure 1. *Epigenetic hypermethylation of CpG islands carried out by DNA methyl-transferase. A) Transcription factor attachment to DNA strand induces RNA polymerase to initiate transcription and gene expression. B) Methylation of CpG island prevents transcription factor attachment and subsequent DNA transcription and gene expression.*

among hundreds of unmethylated alleles. MSP-based detection of hypermethylation has been successfully used when obtaining samples from body fluids that surround or drain the organ of interest in patients with solid malignancies [16].

DNA hypomethylation

DNA hypomethylation involves removal of a methyl group from methylated DNA strands in a process conducted by several enzymes called demethylases [15]. DNA hypomethylation occurs early in human carcinogenesis and is associated with genetic instability in cancer cells. It leads to transcription activation and increased expression of cancer-promoting genes [17]. It has been proposed that global DNA hypomethylation promotes malignancy through reactivation of transposable elements and loss of imprinting (LOI). Ludgate et al. demonstrated a proportional hypomethylation of satellites genes (Sat α and Sat 2 DNA) in LOI-subtype of Wilms tumor [18].

Histone modification

Histones are proteins around which the double stranded DNA is coiled, giving rise to a structure known as a nucleosome. A histone octamer is a group of four pairs of histone molecules (H2a, H2b, H3 and H4). A nucleosome is therefore formed by a histone octamer surrounded by DNA. This is the basic unit of eukaryotic chromatin. There are essentially two types of chromatin: heterochromatin and euchromatin. Heterochromatin is highly condensed and is thus difficult to transcribe, whereas euchromatin is loosely packed and therefore easily transcribed. DNA and histones can be modified in order to silence or activate genes. As a result, the translated or untranslated protein can cause the activation or silencing of other genes in a process that resembles a chain effect. Histone modification can occur through methylation, acetylation and/or phosphorylation.

A number of studies have found that loss or gain-of-function of histone-modifying enzymes, including histone lysine methyltransferases, is pathogenic in several types of cancer. Aberrant activity of histone-modifying enzymes might result in altered chromatin configuration and the subsequent disruption of normal transcriptional programs, pushing the cell towards malignant activity [19]. A recent review identified that histone modifications at *H3K4me2* and *H3K18Ac* (associated with active transcription) and *H3K9me2* (associated with gene repression) were able to predict disease outcome. However, only *H3K9me2* was found to be a prognostic indicator of poorer outcome in metastatic RCC patients [20].

MicroRNA regulation

Regulation of post-transcriptional gene expression takes place through the epigenetic mechanism of RNA interference. miRNAs are a class of small noncoding RNAs that regulate this process. Within tumor cells, dysregulation of miRNA expression can occur, affecting its differentiation, proliferation and apoptosis. The overexpression of miRNA in tumor cells has been termed oncogenic, whereas miRNA that shows reduced expression is referred to as a tumor suppressor [21]. Down-regulation of miRNA expression has been identified as a feature in ccRCC in previous studies. Similarly, dysregulated miRNAs in RCC, such as miR-210, miR-141 and 200c, have been found to give rise to renal carcinogenesis [22].

Huang et al. reported that the down-regulation of miR-30c promotes epithelial mesenchymal transition (EMT) in human renal cell carcinoma [23]. EMT is a transient process in which epithelial cells acquire a mesenchymal phenotype, that is, the loss of intercellular adhesions maintained by a group of proteins called cadherins and the subsequent increase in cell motility. As malignant cells initially proliferate at a higher rate than angiogenesis, a microenvironment of hypoxia is generated. As a result of this, hypoxia-inducible factors (HIF) are released [24]. Huang et al. found that the down-regulation of miR-30c could be induced by hypoxia *via* HIF. The subsequent repression of miR-30c expression results in the reduction of E-cadherin production and promotion of EMT. Conversely, overexpression of miR-30c was found to inhibit EMT in RCC [23]. In order to characterize miRNAs, two approaches are used: studying expression of known miRNAs by hybridization-based techniques or discovery of novel miRNAs molecules by cloning and sequencing [25].

Clinical application

Diagnosis and prognosis

Tumor markers have become a useful tool in diagnosis of malignancy, as they are typically simple to utilize, readily measured in blood and urine samples and do not require invasive procedures. Ideally, a tumor marker should have the following features: be repeatedly present only in cancer patients, correlate with disease stage and response to treatment, and be easily measured [26]. Several proteins have been investigated as potential tumor markers for RCC including carbonic anhydrase 9 (*CAIX*), hypoxia-inducible factor 1-α (*HIF1α*), vascular endothelial growth factor (*VEGF*) and C-reactive protein (*CRP*). Tumor markers are not routinely used

in clinical practice; however they can be helpful in aiding the diagnosis of problematic cases. Lucarelli et al. recently reported on the association between tumor marker levels of *CA 15-3, CA 125* and *β-2 microglobulin* in 332 patients who underwent nephrectomy for RCC. They found that these markers were increased preoperatively in many patients and that serum levels of *CA 15-3* correlated with tumor grade and stage [27]. Gerashchenko et al. reported the association between down-regulation of *NKIRAS1* gene expression and RCC. They found that high grade tumors showed lower expression of *NKIRAS1* compared to low grade tumors [28]. One study reported the use of hypermethylated in cancer 1 (*HIC1*) as a possible marker to enhance individualized therapy and risk stratification in RCC patients. They found that (*HIC1*) hypermethylation was associated with reduced recurrence-free survival [29]. Patricio et al. reported that the transcription factor *paired-box 2 (PAX-2)*, which is intensely expressed during early stages of kidney development, might be useful as a tool to discriminate chromophobe RCC (cRCC) from oncocytoma. They also found that *PAX-2* expression was significantly lower in cRCC compared to ccRCC and pRCC [30]. Specific DNA alterations and molecules in intracellular pathways might be used as potential biomarkers for RCC prognostication. The majority of them are not used in clinical practice yet [3]. Xiao et al. reported the association between *fibulin-1* (an extracellular matrix glycoprotein) down-regulation and the progression of RCC. They suggest that down-regulation of *fibulin-1* through promoter hypermethylation correlated with RCC progression and that by restoring

Table 2. *Clinical and *preclinical trials: HDAC inhibitors*

Protocol	Compound	Methods	Results
Chalret du Rieu Q, et al. 2014 [48]	Abexinostatat	125 patients with either solid tumors or lymphoma were included. Model-derived Recommended Doses (MDRD) of abexinostat (a HDAC inhibitor) was determined from simulations of different administration schedules	Simulation results demonstrated that abexinostat administration during the first 4 days of each week in a 3 week cycle led to a higher MDRD compared to the other administration schedules tested
Guzman ML, et al. 2014 [49]	AR-42 (OSU-HDAC42)	Using an in silico gene expression--based screen for compounds evoking transcriptional effects AR-42 was identified	AR-42 causes potent and specific cell death of leukemia stem cells but not to normal hematopoietic stem and progenitor cells
*Santo L, et al. 2012 [50]	ACY-215 with Bortezomib	2 different xenograft SCID mouse models were used. Human multiple myeloma was injected subcutaneously and luciferase-expressing human multiple myeloma was injected IV	Tumor growth was significantly delayed and overall survival was significantly prolonged in animals treated with the combination therapy of ACY-1215 and bortezomib
Amiri-Kordestani L, et al., 2013 [51]	Romidepsin	Open label, single arm 3plus 3 dose escalation study. Romidepsin was administered in as a four-hour infusion on days 1, 3 and 5 of a 21 days cycle	28 patients with solid tumors including 11 patients with thyroid cancer. Romidepsin was found tolerable and resulted in histone acetylation
*Kurundkar D, et al. 2013 [52]	Vorinostat	Human epidermoid carcinoma cells were obtained and injected subcutaneously in female athymic mice. Mice with palpable tumors were divided into two groups for the study	Vorinostat reduces tumor growth, proliferation and induces apoptosis in xenograft tumors
*Qi YF, et al. 2014 [53]	Suberoylanilide hydroxamic acid (SAHA) also known as Vorinostat	A series of microarray experiments were conducted to investigate tumor cell-selective proapoptotic transcriptional responses induced by Suberoylanilide Hydroxamic Acid (SAHA)	Analyses indicated that SAHA selectively disrupted the DNA damage response, cell cycle, p53 expression and mitochondrial integrity of tumor samples to induce selective tumor cell apoptosis
*Deng C, et al. 2014 [54]	LY2409881 with Romidepsin	Synergy of LY2409881 with other drugs active in lymphoma was determined by calculating relative risk ratio (RRR) and combination index (CI)	LY2409881 suppressed the activity of the NF-kB subunit p65 in lymphoma cells treated by the HDAC inhibitor romidepsin, underlying a potential mechanism of the marked synergy observed of these two drugs

fibulin-1 expression, RCC cell growth was significantly inhibited. Therefore, *fibulin-1* functions as a novel candidate tumor suppressor gene in RCC [31]. Cell adhesion molecules (CADMs) comprise a protein family that participates in cell polarity maintenance and tumor suppression. He et al. reported in their study that *CADM2* is a tumor suppressor gene that prevents progression, invasion and metastasis of renal cancer, and that its expression is silenced at least in part through promoter hypermethylation [32].

A recent study indicates that epigenetic silencing of *NaK--β gene (ATP1B1)* through promoter hypermethylation contributes to RCC initiation and disease progression. They also showed that knockdown of the *(VHL)* tumor suppressor gene in RCC cell lines resulted in *ATP1B1* promoter hypermethylation [33]. Morrissey et al. recently demonstrated that the urine biomarkers of ccRCC and pRCC, specifically aquaporin-1 (AQP1) and perlipin-2 (PLIN2), correlate with tumor size and stage. Their results showed that urine AQP1 concentrations can distinguish ccRCC and pRCC from controls with a sensitivity and specificity of 100% and 100%, respectively. PLIN2 concentrations displayed a sensitivity and specificity of 92% and 100%, respectively [34]. Methylation of the microRNA (mir)-124-3 CpG island was suggested as an independent prognosticator for ccRCC by Gebauer et al. Their analysis demonstrated that increased methylation of a subregion of mir-124-3 was associated with adverse clinicopathologic parameters including metastasis, higher grade, greater tumor size and risk of recurrence [35].

Recent studies have suggested that circulating miRNAs could be used as biomarkers for diagnosis and prognosis in cancer patients. Iwamoto et al. reported that up-regulation of serum miR-210 may occur in the early stage of ccRCC and can therefore be used as a biomarker for early ccRCC in humans [36]. Zhao et al. indicated that miR-187 was down-regulated in both tumor tissue and plasma of ccRCC patients. Interestingly, they found that all patients with high miR-187 expression were alive after 5 years whereas among those with low miR-187 expression, 5 year survival was only 42% [37].

Table 3. *Clinical and *preclinical trials: DMNT inhibitors*

Protocol	Compound	Methods	Results
Chen S, et al. 2014 [55]	DC_05, DC_501 and DC_517	By combining docking-based virtual screening with biochemical analysis, a novel compound DC_05, a non-nucleoside DNMT 1 inhibitor, was identified. Through a process of similarity-based analog searching, compounds DC_501 and DC_517 were found to be more potent than DC_05	These three potent compounds significantly inhibited cancer cell proliferation
Fandy TE, et al. 2014 [56]	5-Aza-2'-deoxycytidine (DAC)	Flow cytometry was used for ROS accumulation analyses. DNA methylation was detected by methylation-specific PCR. Western blotting was used for quantitative protein expression analysis	5-Aza-2'-deoxycytidine (DAC) induced cell cycle arrest and apoptosis in leukemia cells. P53 expression was dispensable for DAC-induced apoptosis.
Winquist, E., et al., 2006 [57].	MG98	Untreated adult patients with measurable MRC were treated with MG98 at a dose of 360 mg/m^2 via 2-h IV infusion twice weekly for three consecutive weeks out of four	No conclusive pattern of decreased DNMT1 activity in peripheral blood mononuclear cells was detected post MG98 treatment. The lack of objective responses observed may be explained by a lack of target effect or the choice of tumor type
*Tikoo K, et al. 2009 [58]	5-azacytidine and cisplatin	Colon cancer was induced in male SD rats. After 6 months of the first month of carcinogen, rats were randomly divided into five groups. 1st and 2nd group received normal saline on day zero. The other 3 groups received intraperitoneal injections of 5-azacytidine, cisplatin and the combination of the two drugs on day zero	5-azacytidine potentiated cisplatin induced antitumor activity by involving decreased expression of pAKT, DNMT1 and an increased expression of p38 in colon tumors

Targets for treatment

Immunotherapy for advanced RCCs, including the administration of interferon-alpha and interleukin-2 (IL-2), has been used as a standard treatment for over 20 years. In addition to immunotherapy and traditional surgical approaches, several molecular targeted therapies have been adapted into clinical practice, including the use of mammalian target such as rapamycin (mTOR) inhibitors and tyrosine kinase inhibitors (TKI), both of which target the VEGF receptor [2]. Since epigenetic changes are heritable and potentially reversible, it is reasonable to use them as potential therapeutic targets [15]. Several molecules have been developed to target epigenetic changes that involve DNA methylation (DNA methyl transferases (DNMT) inhibitors) and histone modification (histone deacetylase (HDAC) inhibitors). Some clinical and preclinical trials of these molecules are showed in tables 2 and 3. The use of chemically modified antisense-oligonucleotides has been explored in the past as a method of miRNA knockdown; however, the biodistribution, biostability and mode of delivery have been an important challenge. The development of alternative and non-conventional methods to target miRNA is therefore necessary [38]. Currently, miRNA inhibitors are offered as vector-based expression clones or synthetic oligonucleotides. Vector-based expression clones exist in lentiviral and non-viral vectors. Transient and stable suppression of the target gene is achieved when the miRNA inhibitor clones bind specifically to the target miRNAs.

DNMT inhibitors

Humans contain three catalytically-active DNA methyltransferases enzymes (DNMT1, DNMT3a, and DNMT3b) which regulate DNA methylation, in addition to an associated regulatory protein (DNMT3L) [39]. As indicated previously, methylation of CpG dinucleotides has been linked to the development of RCC. Therefore, molecules that target DNMTs would appear to be excellent therapeutic agents against RCC. One promising inhibitor of human DNMT1 is MG98. It binds to DNMT1 messenger RNA (mRNA) preventing further processing of the mRNA and reducing cellular levels of DNMT1 [40].

One signaling pathway that has been implicated in the pathogenesis of RCC in recent studies is the Wnt/β-catenin pathway. Studies have shown that promoter hypermethylation of Wnt antagonists results in carcinogenesis through dysregulation of cell proliferation and differentiation. Secreted-frizzled-related protein 2 (sFRP2) is a Wnt antagonist that functions as a tumor suppressor gene. Its expression levels have been found to be down-regulated in renal cancers. A recent study suggested that the use of 5-aza-2´-deoxycytidine (DAC), a DNMT inhibitor that restores sFRP2 expression, induces apoptosis in RCC cells [41].

HDAC inhibitors

Histone modifications include acetylation (transcriptional activation), methylation (activation or repression) and phosphorylation (chromatin structure and function alteration). Aberrant activity of the enzymes implicated in these changes could lead to abnormal chromatin configuration and subsequent development of cancer. Histone deacetylase inhibitors are categorized based on their structure into hydroxamates (vorinostat), cyclic peptides (romidepsin), aliphatic acids (phenylbutyrate) and benzamides (entinostat) [20]. Several new agents are currently under development. A novel HDAC inhibitor called OBP-801, also known as YM753, was reported to induce apoptosis and inhibit cell growth of RCC cells when combined synergistically with the phosphatidylinositol 3-kinase (P13K) inhibitor LY294002, rendering this combination to be a promising treatment for RCC [42].

Several therapeutic benefits of HDAC inhibitors have been described in pre-clinical trials, but unfortunately, this has not translated into clinical trials to date. One reason could be the development of acquired resistance due to long-term drug treatment. A recent study showed how the prolonged use (12 weeks) of a HDAC inhibitor (valproic acid) was associated with drug resistance when compared with a short 2 weeks treatment. They found that the chronic use of valproic acid enables the reactivation of Akt, also known as Protein Kinase B, which may be involved in resistance development [43].

CONCLUSIONS

The three main epigenetic mechanisms implicated in carcinogenesis include DNA methylation, histone modifications and miRNA regulations. Epigenetics has become an important topic in cancer research during the last decade, and its role in the diagnosis, prognosis and treatment of kidney malignancies appears to be a promising field. The clinical application of epigenetics has shown to have the potential to allow for early detection and prognostication in kidney cancer, as well as to provide tools for the introduction of newer therapeutic agents. The feasibility of epigenetic changes to be potentially "reversed" motivates researchers to continue pursuing these targets as treatment options for RCC and other malignancies.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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