Chapter 4
Epigenetics and MicroRNAs in Renal Cancer

Shahana Majid, Sharanjot Saini, Guoren Deng, and Rajvir Dahiya

Abstract Epigenetic states are orchestrated by several converging and reinforcing signals, including DNA methylation, histone modifications and non-coding RNAs. Growing evidence indicates that acquired epigenetic abnormalities participate with genetic alterations to cause cancer. In this review we describe recent advances in the field of cancer epigenomics and microRNAs (miRNAs) with special emphasis on renal cancer. We discuss whether epigenetic changes are the cause or consequence of cancer initiation and the use of epigenetic biomarkers and miRNAs for cancer diagnosis or prognosis. Finally we address the potential of epigenetic based anti-cancer therapeutic strategies.

Keywords Epigenetics • MicroRNA • Renal cancer

4.1 Background

The term 'epigentics' was originally coined by Conrad Waddington in 1942 for the molecular mechanisms that convert genetic information into observable traits or phenotypes during development [1]. By contrast, Arthur Riggs et al. defined epigenetics as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" [2]. The term may be currently defined as the mechanisms that initiate and maintain heritable patterns of gene function and regulation without affecting the sequence of the genome [3]. The sum total of all epigenetic information is termed the 'epigenome' and comprises

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some of the instructions directing the genome to express genes at particular places and times [4, 5]. Unlike the genome, the epigenome is highly variable between cells and fluctuates in time according to conditions even within a single cell. Each of us has essentially one genome, however each cell type in each individual is believed to have a distinct epigenome that reflects its developmental state [6]. The epigenetic state of a cell is affected by developmental as well as environmental influences that may leave epigenetic traces which the cell remembers, referred to as cellular memory [7]. Thus the epigenome provides a crucial interface between the environment and the genome. Recent breakthroughs in the understanding of epigenetic mechanisms provide evidence that they are fundamental to the regulation of many cellular processes, including gene and microRNA expression, DNA-protein interactions, suppression of transposable element mobility, cellular differentiation, embroygenesis, X-chromosome inactivation and genomic imprinting [8]. The disruption of epigenetic changes underlies a wide variety of pathologies including cancer [9]. The cancer epigenome is characterized by global changes in DNA methylation including hypomethylation, promoter specific hypermethylation, histone modification, chromatin-modifying enzyme expression profiles and global dysregulation of non-coding microRNAs (miRNAs). These aberrations confer a selective growth advantage to neoplastic cells, apoptotic deficiency and uncontrolled cell proliferation, leading to cancer initiation and progression. For didactic purposes, epigenetic mechanisms may be grouped into DNA methylation, histone modification and remodeling and miRNAs. In this review, we will describe these mechanisms with an emphasis on alterations of the epigenome taking place in renal cancer.

4.2 DNA Methylation

Aberrant DNA methylation is the best characterized cancer-related epigenetic modification. DNA methylation occurs predominantly at the symmetrical dinucleotide CpG sites [10] that are scattered throughout the genome at a lower-than-expected frequency. However, in certain areas of the genome, a high concentration of CpG dinucleotides is found, and are referred to as “CpG islands” (CGIs) [11]. In a normal differentiated cell, CpG loci disseminated across the genome are highly methylated, whereas most promoter CGIs are protected from methylation inside their boundaries [11]. In general CGI methylation is associated with gene silencing. Gene silencing associated with CGI promoter methylation may be due to restricted access of transcription factors or binding of methylcytosine-binding proteins (MBD), which cooperate with DNMTs and histone deacetylases (HDACs) [12]. An important role in the regulation of gene expression has also been credited to low density CpG regions located in the vicinity of CpG islands, the so-called “CpG island shores” [13, 14]. These are sequences up to 2 kb distant from CpG islands, that are associated with gene expression. Remarkably, methylation patterns at CpG island shores are mostly tissue-specific and cancer-associated alterations in these patterns occur at sites that vary normally in tissue differentiation [14]. Differentially

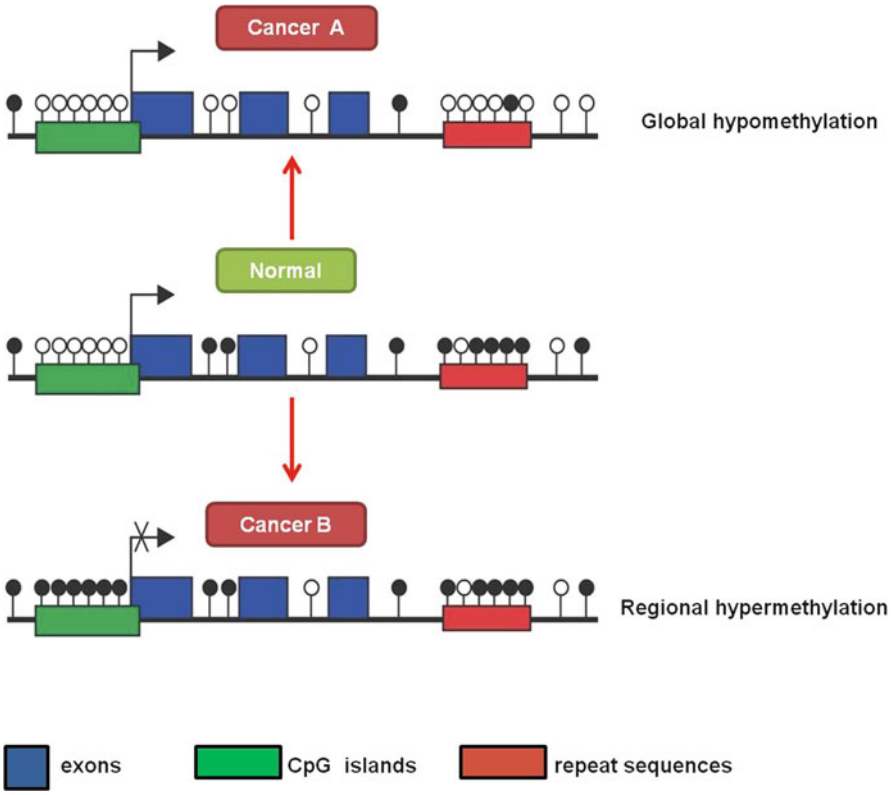

Fig. 4.1 Two epigenetic pathways of transformation from normal cells to cancer cells. In normal cells, DNA is unmethylated in CpG islands, while in repeat sequences and CpG sparse regions, DNA is heavily methylated. When DNA is less methylated in repeat sequences and CpG sparse regions, cells are transformed (Cancer A with global hypomethylation). If DNA is heavily methylated in CpG islands, cells are also transformed (Cancer B with regional hypermethylation)

methylated CpG island shores are sufficient to distinguish between specific tissues and are conserved between human and mouse [13, 15]. Aberrations in DNA methylation include both global and gene-specific hypomethylation as well as gene-specific CpG island promoter hypermethylation [3, 16] (Fig. 4.1). Since global DNA hypomethylation and promoter-specific hypermethylation can be commonly observed in benign neoplasias and early-stage tumors, it is becoming apparent that epigenetic deregulation may precede the classical preliminary transforming events such as mutations in tumor suppressors, protooncogenes and genomic instability [17]. These aberrations have also have been considered to be the earliest events in the process of tumorigenesis [18]. The impact of gene-specific alterations in DNA methylation depends on the function of the affected gene and the type of alteration. Whereas promoter hypomethylation may cause activation of proto-oncogenes, hypermethylation induces silencing of cancer-related genes with tumor suppressive properties [18]. On the other hand, genome-wide hypomethylation may lead to

Table 4.1 Genes and microRNAs methylated in renal cell carcinoma

Gene	References
WNT7a	[27]
TCF21	[28]
SLC34A2	[29]
OVOL1	[29]
DLEC1	[29]
TMPRSS2	[29]
SST	[29]
BMP4	[29]
GATA5	[30]
Rap1GAP	[31]
KLHL35	[32]
QPCT	[32]
SCUBE3	[32]
ZSCAN18	[32]
CCDC8	[32]
FBN2	[32]
ATP5G2	[32]
PCDH8	[32]
CORO6	[32]
DLEC1	[33]
miR-34a	[34]
miR-34b/c	[34]
miR-9	[35][a]

[a]For other methylated genes in RCC, please see Ref. [36]

genomic instability in repetitive sequences, especially at pericentromeric regions, predisposing to abnormal recombination, facilitating translocations, deletions, and chromosomal rearrangements [19–21].

Renal cell carcinoma (RCC) is genetically and histopathologically a heterogeneous disorder. The most common subtype of RCC is clear cell RCC (ccRCC; approximately 75 %) and the next most frequent subtype is papillary RCC (pRCC; approximately 15 %) [22]. The most frequent genetic abnormality in ccRCC is inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene [23] and promoter methylation of tumor suppressor geness (TSGs) is common in both subtypes of RCC. The *VHL* and $p16^{INK4a}$ TSGs are inactivated by promoter hypermethylation in up to 20 % of clear cell [24] and 10 % of all RCC [25]. The *RASSF1A* and the *Timp-3* genes are hypermethylated in 27–56 % [26] and 58–78 %, of primary RCCs respectively [26]. Table 4.1 provides an overview of the commonly methylated genes in renal cancer based on the published reports. A survey of published work in 2010 by Morris and Maher [36] has identified 58 genes that are methylated in RCC and 43 of these genes had a mean combined methylation/mutation rate of over 20 % (Ref. [36]). Cancer genome projects such as TCGA (http://cancergenome.nih.gov/l) and CAGEKID (http://www.icgc.org/icgc/cgp/65/812/817) have elected to define the mutational status and methylation profile of RCC. Hence large amount of data will

be available to identify frequently methylated genes in RCC in the near future. Our group has also reported that various genes such as DNA mismatch repair genes [37], E-cadherin [38], gamma-catenin [39] and BTG3 [40] are silenced through promoter hypermethylation in renal cancer. We have also published extensively on the promoter methylation status of genes involved in the Wnt signaling pathway in renal cancer. Oncogenic activation of the Wnt pathway drives expression of genes that contribute to proliferation, survival and invasion. Inhibitors of this pathway can be divided into two functional classes, sFRP proteins that bind directly to Wnt and prevent its binding to frizzled receptor and the Dickkopt (DKK) proteins which bind to LRP component of the Wnt receptor complex. The *sFRP-1, sFRP-2, sFRP-4, sFRP-5* and related Wif genes are all frequently methylated in RCC [41–44], as are the DKK genes [45, 46]. An interesting finding by our group is that *sFRP1* is unmethylated/hypomethylated and thus over-expressed in metastatic renal tumors [47] compared to primary tumors where in its expression is attenuated by promoter hypermethylation [41]. Another study from our group by Yamamura et al. [48] challenged the Wnt inhibitory role of *sFRP2* and reported that overexpression of *sFRP2* activates the canonical Wnt pathway, promoting cell growth through diverse signaling cascades in renal cancer cells [48].

4.3 Chromatin Remodeling and Histone Modifications

The coiling of DNA around nucleosome particles is the basis for organization of eukaryotic genomes. Each nucleosome encompasses ~147 bp of DNA wrapped around an octamer of histone proteins. The core histones H2A, H2B, H3 and H4 bind together (two H2A-H2B dimers and one H3-H4 tetramer) to form the nucleosome. The core histones are small basic proteins containing a globular domain and a flexible charged NH2 terminus known as the histone tail [49]. Regulation of gene expression occurs through posttranslational covalent modifications of the histone tails including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization, and ADP ribosylation [49, 50]. Generally certain histone modifications such as acetylation or phosphorylation are thought to change chromatin structure by altering the net positive charge of the histone proteins, thereby making the underlying DNA sequence accessible [51]. Alternatively, histone modifications can be recognized by specific protein domains (e.g., bromodomains, Tudor domains, chromodomains), which in turn might enforce or stabilize the recruitment of additional factors [52, 53]. Posttranslational modifications to histone tails govern the structural status of chromatin and the resulting transcriptional status of genes within a particular locus. These modifications are reversible and controlled by a group of enzymes including histone acetyltransferases (HATs) and deacetylases (HDACs), methyltransferases (HMTs), demethylases (HDMs), kinases, phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligases and proteases which add and remove these modifications [8, 49]. In relation to transcriptional state, the human genome can be roughly divided into two distinct chromatin conformation states: euchromatin,

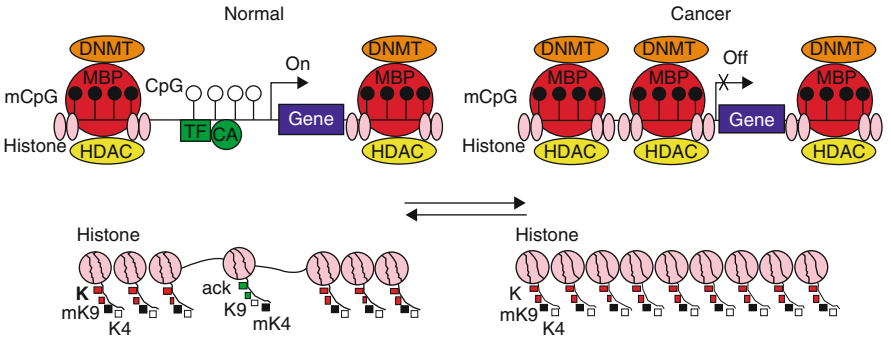

Fig. 4.2 DNA methylation status in the promoter and the related chromatin structure. In normal cells, the CpG sites adjacent to transcription start site are unmethylated. The transcriptional machinery is activated by the binding of transcriptional factor (TFs) and co-acting factors (CAs) in this region. The gene promoter shown on the *upper left* is transcriptionally active. In upstream and downstream regions, DNA is methylated by DNA methyltransferases (DNMTs). In these regions, methylcytosine-binding proteins (MBPs) that bind to methylated CpG sites recruit histone deacetylases (HDACs) and histone methyltransferases to form a complex. *Left bottom* shows the related chromatin structure around the transcriptionally active, unmethylated promoter. The lysine residues in the tails of histone H3 are acetylated (acK). Lysine 4 is methylated (mK4) and lysine 9 is unmethylated (K9). These changes contribute to open and relaxed conformation of the chromatin allowing key components of the transcription apparatus accessible to the promoter. In the upstream and downstream regions, the lysine residues are deacetylated (K), demethylated (K4) and methylated (mK9) respectively and the chromatin structure have closed and dense conformation. In cancer cells, shown in the *upper right*, DNA methylation spreads toward the promoter regions near the transcriptional start site, resulting in transcriptional silencing. These events result in closed and dense chromatin conformation making it difficult for the key components of gene transcription apparatus to bind to the promoter

which has an open structure and is transcriptionally active and heterochromatin, which is densely compacted and transcriptionally inert [54]. Euchromatin is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79. In contrast heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation [8, 54]. The notion of heterochromatin as transcriptionally inactive has been challenged by the discovery of numerous noncoding RNAs (ncRNAs) derived from heterochromatic loci [55]. Well-known examples of this phenomenon in humans are the ncRNAs *XIST* and *HOTAIR* [56, 57]. Histone modifications are predictive for gene expression as actively transcribed genes are characterized by enriched levels of H3K4me3, H3K27ac, H2BK5-azacytidine (H2BK5ac) and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene [58]. Therefore histone modifications influence chromatin structure which plays an important role in gene regulation and carcinogenesis (Fig. 4.2).

Genome-wide studies have revealed that various combinations of histone modifications in a specific genomic region can lead to a more ‘open’ or ‘closed’ chromatin structure resulting in the activation or repression of gene expression. Disruption of normal patterns of histone modifications is a hallmark of cancer [12, 59]. One of the most characteristic examples is the global reduction of H4K20 trimethylation (H4K20me3) and H4K16 acetylation (H4K16Ac), along with DNA hypomethylation,

at repeat sequences in many primary tumors [12]. Furthermore, genes encoding for histone-modifying enzymes have been also reported to be mutated in ccRCC [60]. Mutated genes have been implicated in chromatin regulation through nucleosome repositioning and histone tail modification. *PBRM1*, which was found to be mutated in nearly 40 % of human RCCs [61, 62], is a component of the Polybromo BRG1-associated factor complex (PBAF, SWI/SNF-B). PBAF, like SWI/SNF, functions as a nucleosome remodeler and was shown to be involved in transcriptional regulation (24–26). Less common mutations were also identified in two methyltransferases, SETD2 and MLL2, and two demethylases, UTX (KDM6A) and JARID1C [KDM5C Ref. [60]]. Deletion of chromosome 3p is a common finding in ccRCC associated with the loss of VHL at 3p25 and can also affect SETD2 and PBRM1, which are located at 3p21 [63]. SETD2 mediates the trimethylation of H3K36 [64], a histone mark that is placed during transcription and may be important for maintaining faithful transcription [65], whereas MLL2 mediates H3K4me3, a mark associated with active transcription. UTX demethylates H3K27me3 [66, 67], a histone mark associated with repressed chromatin. Of interest, UTX associates with MLL2 [68], suggesting that demethylation of repressive modification is associated with transcriptional activation. The hypoxia response pathway has been shown to have a direct effect on histone modification. HIF upregulation is a feature of ccRCC and it was shown to activate several chromatin demethylases, including JMJD1A (KDM3A), JMJD2B (KDM4B), JMJD2C (KDM4C), and JARID1B (KDM5B), all of which are directly targeted by HIF [69–71]. Indeed, both JMJD1A and JMJD2B were found to be elevated in a RCC cell line with loss of VHL function [70], and the expression of JMJD1A was reported to be higher in RCC cancer tissue surrounding blood vessels, suggesting that JMJD1A is involved in tumor angiogenesis [72]. Reexpression of VHL in VHL-deficient cell lines increased H3K4me3 levels associated with decreasing levels of JARID1C, a target of HIF2a [62]. Silencing of JARID1C in VHL-deficient tumor cells augmented tumor growth in a xenograft mouse model, suggesting that JARID1C acts as a tumor suppressor. In contrast, hypoxia may increase methylation through HIF-independent mechanisms. Like HIF prolyl hydroxylase (PHD, EGLN3), histone demethylases are members of the dioxygenase superfamily, which requires oxygen as well as iron and 2-oxoglutarate for activity [73, 74]. In a manner analogous to stabilization of HIF via decreased hydroxylation, hypoxia was shown to suppress JARID1A (KDM5A) activity, resulting in increased H3K4me3 levels [75]. This suggests the hypothesis that loss of demethylases (and, by analogy, increased histone methylation) is part of a hypoxia phenotype that is selected for in RCC. This hypoxia phenotype, which is mimicked by *VHL* loss, would also be mimicked by loss of histone demethylase activity, which is a high-frequency event in RCC. Chromatin organization also influences HIF function. Studies of HIF induced under conditions of hypoxia showed preferential targeting of HIF to previously nucleosome depleted chromatin regions [76]. Moreover, the coexpression of SWI/SNF components BRG1, BAF170, and BAF57 augmented HIF activity from an HIF responsive reporter [77]. The extent to which mutations of epigenetic regulators influence chromatin or HIF targeting remains unknown.

4.4 MicroRNAs

MicroRNAs are small, non-protein-coding sequences thought to regulate >90 % of human genes by targeted repression of gene transcription and translation [78]. These endogenous, silencing RNAs have been shown to play important roles in development and differentiation [79, 80], cellular stress responses [81], and growing evidence has strongly implicated the involvement of miRNAs in carcinogenesis [82–84]. Specific subsets of miRNAs have also been shown to be dysregulated in various solid tumors [85, 86]. Due to their tremendous regulatory potential and tissue-specific and disease-specific expression patterns [87, 88], there is increasing evidence that miRNA expression profiles could be indicative of disease risk.

DNA hypermethylation of CpG sites within CpG islands is known to lead to the inactivation of many tumor-suppressive miRNAs [89–91]. One of the most common causes of tumor-suppressor miRNA loss is silencing of their primary transcripts by CpG-island hypermethylation [92–96]. The DNA methylation profile of tumors is useful to define tumor type, clinical prognosis and treatment response [19, 20]. Epigenetic silencing of miRNAs is also involved in the acquisition of an invasive phenotype and the development of metastasis [93]. Dysregulation of miRNA expression seems to be pivotal for RCC development and progression. Table 4.2 presents a list of miRNAs that are dysregulated in RCC. Depletion of tumor suppressor genes or upregulation of oncogenes has also been correlated with dysregulated expression of miRNAs in RCC. Our group has reported that several tumor suppressor miRNAs such as miR-1826 [102], miR-708 [100], miR-205 [104], miR-584 [105] are attenuated in RCC, where as oncogenic miR-21 was overexpressed [108]. There are controversial reports about the status of miRNA-34a in RCC. One study reported that inactivation of miR-34a correlates with its methylation status as they found methylation frequency of 58 % in RCC [34]. "Whereas in contrast, Liu et al. [109] reported increased levels of mir-34a caused loss of function of tumor suppressor *SFRP1* [which again is a controversial tumor suppressor in RCC [47]] indicating its oncogenic potential" [109]. However no functional analysis was

Table 4.2 MicroRNAs and their targets in renal cell carcinoma

microRNAs	Function	Target gene	References
miR-99a	Tumor suppressor	mTOR	[97]
miR-138	Tumor suppressor	Vimentin	[98]
miR-204	Tumor suppressor	MAP1LC3B	[99]
miR-708	Tumor suppressor	Survivin	[100]
miR-1	Tumor suppressor	transgelin-2	[101]
miR-133a	Tumor suppressor	transgelin-2	[101]
miR-1826	Tumor suppressor	CTNNB1, MAP2K1	[102]
miR-34a	Tumor suppressor	c-Myc	[103]
miR-205	Tumor suppressor	Src kinase	[104]
miR-584	Tumor suppressor	ROCK1	[105]
miR-23b	Oncogenic	Proline oxidase	[106]
miR-21	Oncogenic	PTEN	[107]

performed in either study. A recent study by our group investigated the functional effects of miR-34a in RCC [103]. It reported that overexpression of miR-34a inhibited cell invasion and suppressed the assembly and function of the c-Myc complex that activates or elongates transcription, indicating a tumor suppressor role in RCC [103]. Matching patterns between deregulated miRNAs and chromosomal aberrations have been reported in ccRCC [110]. On the other hand, miRNA deregulation might serve as an alternative mechanism for gene expression alterations due to chromosomal aberrations. This is well illustrated by the miR-204/211 family. Gain of chromosome 3q is a common finding in papillaryRCC that leads to upregulation of several genes including *C3orf58, CCDC50, DTX3L, PLD1, TRIM59, ECT2, RAP2B,* and *SERP1* that are targeted by miR-204/211 [Ref. [109]], whereas in ccRCC, miR-204/211 downregulation might be the mechanism causing upregulation of the same set of genes, since 3q gain is rare in ccRCC [109].

4.5 Interplay Between Epigenetic Factors

There is interplay between histone modifications and DNA methylation and the best example is the relationship between DNMT3L and H3K4. DNMT3L specifically interacts with histone H3 tails, inducing *de novo* DNA methylation by recruitment of DNMT3A, however this interaction is strongly inhibited by H3K4me. Furthermore, several histone methyltransferases have also been reported to direct DNA methylation to specific genomic targets by recruiting DNMTs [111, 112], helping in this way to set the silenced state established by the repressive histone marks. Moreover, histone methyltransferases and demethylases can also modulate the stability of DNMT proteins, thereby regulating DNA methylation levels [113, 114]. On the other hand, DNA methylation can also direct histone modifications. For instance, methylated DNA mediates H3K9me through MeCP2 recruitment [115]. MicroRNAs are also known to target the components of epigenetic machinery such as DNMTs, HDACs and polycomb genes [116]. Whereas, miRNAs may be affected by epigenetic changes, such as methylation of the CGIs and accompanying changes in histone modifications. miR-127 has been found to be attenuated in cancer cells by promoter hypermethylation and by a decrease in acetyl-H3 and methyl-H3K4 [95]. Genome-wide analysis of different cancer types has shown that global expression of miRNAs is influenced by DNA methylation and histone modifications [117].

4.6 Epigenetic and miRNA Biomarkers

Methylated DNA sequences provide attractive options for biomarkers for cancer detection and prognosis including RCC [118]. The last decade has provided an extensive map of the aberrant DNA methylation events occurring in cancer cells, particularly for the hypermethylated CpG islands of tumor suppressor genes (TSG) [19].

Consequently a myriad of DNA methylation-based biomarkers of many types of human neoplasias have been reported. Different RCC subtypes seem to display different gene sets deregulated by promoter hypermethylation [26], and a gene panel (*CDH1*, *PTGS2*, and *RASSF2*) identifying most frequent RCC subtypes in tissue samples has been evaluated [119]. The epigenomic data have helped highlight the unique profile of aberrant DNA methylation that defines each tumor type [120]. Epigenetic biomarkers are of particular interest as non-invasive biomarkers since methylated DNA can be detected from tumor cells sloughed into urine or blood. This has been shown with a three-gene panel (*APC*, *RARβ2*, *RASSF1A*) which detected RCC with high specificity and sensitivity [41, 121]. Moreover, *RASSF1A* promoter methylation might also prove useful for tumor surveillance/monitoring of RCC cancer patients [122]. Methylation of the Wnt pathway genes *SFRP1, SFRP2, SFRP4, SFRP5, DKK3* and *WIF1* have been detected in the serum of patients with corresponding tumor methylation and the frequency of methylation in serum correlated with increased grade and stage [41]. Therefore the detection of RCC-associated TSG methylation by analysis of serum or urine samples could have potential for early detection of RCC and for distinguishing benign and malignant renal cancers. Promoter hypermethylation of some genes has been associated with clinical and pathological features of tumor aggressiveness and also with prognostic relevance. Aberrant promoter methylation of *APAF1, DAPK1* and *GREM1* [123] has been associated with aggressive forms of RCC. Moreover, promoter methylation of *APAF1*, *DAPK1* [124], *JUP* [39], *PTEN* [125], *UCHL1* [126], *DAL1-4.1B/EPB41L3* [127] *BNC1* and *COL14A1* [128] have been associated with poorer survival, and most of them (*JUP*, *APAF1*, *DAPK1*, *PTEN*, *DAL1-4.1B*, *BNC1*, and *COL14A1*) retained independent prognostic value in multivariate analysis [39, 124, 128]. Clearly it is important that there should be additional studies of potential methylated biomarkers in tumor tissues and urine and/or blood with the ultimate aim of producing a panel of biomarkers that will enable non-invasive detection, molecular staging and prediction of prognosis. As the number of potential methylated TSG biomarkers increases, it will be of great importance to assay these in a standardized manner in prospective studies to establish their clinical utility.

Genome-wide studies of histone modifications have been performed to characterize the chromatin of malignant cells by establishing the overall profile of histone modifications in cancer cells. Signatures of histone modifications patterns, such as trimethyl-H3K9, are associated with patient prognosis in acute myeloid leukemia [129]. Silencing of genes marked by trimethyl-H3K27 in the absence of DNA methylation has also been reported [130]. Several histone modifications have been associated with poor prognosis in RCC, including low H3K4me2, H3K18ac, and H3K9me2 [131]. H3K4me1–3 levels were also found to be inversely correlated with Fuhrman grade, stage, lymph node involvement and distant metastases, and an H3K4me score was an independent factor for RCC progression free survival [132]. Similar observations have been made for global H3Ac and H4Ac levels, as well as for H3K9Ac levels in RCCs treated with partial nephrectomy [133], whereas H3K18Ac levels were an independent predictor of RCC progression after surgery [134].

The use of genome wide approaches has enabled the production of miRNA fingerprints in a range of tumors and the identification of new potential biomarkers to distinguish tumor tissue from its normal counterpart. From a clinical point of view, miRNAs have great potential as diagnostic and therapeutic agents. Owing to the tissue specificity of miRNAs, they have become a useful tool for defining the origin of tumors in poorly differentiated cancers [135]. Prognosis and survival of patients depends on the cancer stage at diagnosis and miRNA signatures have been reported to be useful tools for early diagnosis of cancer [136, 137]. Differential miRNA expression patterns between neoplastic and non-neoplastic renal tissues, as well as among different renal tumor subtypes have been described. Discrimination between ccRCC and normal kidney tissue have been described with a panel of nine miRs (miR-21, miR-34a, miR-142-3p, miR-155, miR-185, miR-200c, miR-210, miR-224, and miR-592) [Ref. [138]], a combination of miR-141 and miR-155 [139] or by differential expression of miR-92a, miR-210, and miR-200c [140]. For a more clinical perspective with the aim of supporting diagnosis, a stepwise decision tree was created to differentiate between kidney cancer subtypes and oncocytoma, depending on miRNA signatures. This method is valuable in small biopsy samples and in cases where morphological assessment is not sufficient for diagnosis [141]. Unsupervised hierarchical cluster analysis of miRNA microarray data showed that tumors derived from the proximal and distal nephrons can be distinguished by their miRNA profile [140]. The differential expression patterns of miRNAs can also be used to subclassify renal cancer. In ccRCC 23 miRNAs are differentially expressed (let-7e, let-7f, let-7g, miR10b, miR-124, miR-126, miR-138, miR-140-5p, miR-142-5p, miR-144, miR-184, miR-200c, miR-203, miR-206, miR-210, miR-218, miR-27a, miR-27b, miR-335, miR-373, miR-378, miR-92a, miR-98. However, some miRNAs are characteristic of sporadic ccRCC (let-7c, let-7d, miR-1, miR-100, miR-10a, miR-148b, miR-191, miR-199a-3p, miR-19a, miR-215, miR-29b, miR-30c, miR-363, miR-9) and others of hereditary RCC (let-7a, miR-125a-5p, miR-125b, miR-143, miR-146b-5p, miR-15b, miR-17, miR-193a-5p, miR-193b, miR-196a, miR-20b, miR-214, miR-23b, miR-32, miR-372) [61]. miRNA levels in sera of RCC patients and healthy controls, identified miR-1233 as a promising biomarker for RCC detection and monitoring [142]. Altered levels of miRNA might also provide prognostic information. Whereas miR-155 and miR-21 expression in ccRCC tumors has been found to correlate with tumor size [143], higher miR-210 levels were found in tumors displaying higher Fuhrman grade [140]. In ccRCC, overexpression of miR-32, miR-210, miR-21, and miR-18a correlated with poor survival [143, 144]. Lower miR-106b levels were associated with metastatic disease and poorer relapse-free survival [145]. High miR-210 expression was also found in tumors with lymph node metastasis [140], suggesting unique miRNA signatures in metastatic RCC, distinct from those of primary tumors [146]. Khalla et al. [147] compared distant metastases with primary tumors and found a distinct miRNA signature in metastases. Some of the primary tumor samples clustered together with the distant metastasis, suggesting that these primary tumors have a metastasis-specific signature [147]. Because miRNAs can be easily detected and quantified in blood, serum assays based on metastasis-associated miRNAs may be of value.

In addition, Lin et al. [148] identified 12 SNPs in miRNA-related genes that are significantly associated with recurrence or survival and found a cumulative effect of multiple SNPs with recurrence. Taken together, additional studies in large patient cohorts are necessary to validate the potential use of miRNAs as diagnostic/prognostic biomarkers.

4.7 Epigenetics as Consequence or Cause of Cancer Initiation

Cancers are caused by accumulative mutations in the genes [149]. Mutations cause rearrangements of large chromosomal regions, which confer the cells with growth advantage under selection pressure due to abnormal expression of oncogenes [149, 150]. The clonal expansion of the mutated cells leads to genomic instability and global demethylation, while the cell machinery progressively shuts down the anti-survival genes by hypermethylation. Thus mutations cause genomic instability, which precedes methylation changes. By contrast, congenital disorders such as ICF syndrome and Rett syndrome involve genes that encode the methylation machinery of the cell such as *DNMT3B* (ICF syndrome) and *MECP2* (Rett syndrome), but these disorders do not predispose to cancer. Thus, epigenetic changes were thought to be a consequence of altered gene expression rather than causal [151]. Further, activation of tumor suppressor genes by 5-aza-2′-deoxycytidine or *DNMT1* knockout may not be stable, as has been shown for both *MLH1* [152] and *p16* [153], suggesting that the altered methylation might be a consequence rather than a cause of gene silencing. Thus a key barrier to the acceptance of epigenetic alterations as a cause rather than a consequence of cancer has been the lack of well-defined human pre-neoplastic disorders that are caused by epigenetic mutations. However the discovery of the mechanisms of Beckwith-Wiedemann syndrome (BWS) provides a good example of constitutional epigenetic alterations linked to cancer risk. BWS was shown to have various molecular causes, including loss of imprinting (LOI) of *IGF2* [154] or point mutations in the *CDKNIC* [155] gene or epigenetic lesions in the nearby antisense RNA *LIT1*. Furthermore, cancer predisposition might be specifically associated with LOI of *IGF2* and hypermethylation of *H19* [156]. In a large registry of patients with BWS gain of methylation at *H19,* presumably resulting in biallelic expression of *IGF2*, was found to be specifically and statistically associated with cancer risk [157]. BWS leads to an 800 fold increased risk of embryonal tumors such as Wilm's tumor of the kidney and rhabdomyosarcoma [158]. LOI of *IGF2* is specifically associated with increased cancer risk in children with BWS. Thus the epigenetic change precedes cancer and confers risk for cancer, a strong argument for causality. Another study showed that aberrant changes in the epigenome could indeed lead to cancers that do not display genomic instability [159]. *Snf5* is a tumor suppressor gene and a core component of the chromatin remodeling complex SWI/SNF whose inactivation is detected in several types of tumors [160, 161], including the highly invasive

malignant rhabdoid tumors (MRTs) [162]. Differing from most other tumors where the chromosomes are usually fragmented, MRTs often display an intact genome. The authors generated *Snf5*-deficient primary mouse embryonic fibroblasts and showed that tumors derived from these cells were diploid and the cancer phenotype was correlated with the expression of the cell cycle protein cyclin D1, which was epigenetically upregulated by SWI/SNF complexes [159]. An alternative approach to study the relationship between epigenetic changes and transformation is to study the epigenome of pre-cancerous cells. A series of studies on colon cancers found that global hypomethylation as well as regional gene promoter hypermethylation occur in pre-cancerous lesions or even benign colon polyps before they become malignant colon cancers [163–165]. Similar findings have been observed in breast cancers, where normal tissues surrounding the tumors have been detected with aberrant DNA methylation patterns [166, 167]. These observations of methylation patterns change in pre-cancerous cells suggest that the loss in methylation can be an early event that precedes malignancy. Experimental data in mice also support a causal role for epigenetic changes in cancer. When *DNMT1* hypomorphs are crossed with Min (multiple intestinal neoplasia) mice with an *Apc* mutation, they show an increased frequency of intestinal neoplasia and liver cancers [168]. In addition, it has also been shown that global hypomethylation leads to elevated mutation rates [169], suggesting that epigenetic changes may initiate downstream oncogenetic pathways. Studying these model systems may therefore aid our understanding of how epigenetic processes contribute to the process of oncogenic malignancy.

4.8 Epigenetic Therapy

Given that epigenetic modifications are reversible, it seems likely that understanding and manipulating the epigenome may hold promise for preventing and treating common human diseases including cancer. Much attention has been focused on the quest for epigenetic drugs, which restore the normal epigenetic landscape in cancer cells by inhibiting enzymes of the epigenetic machineries. Understanding the mechanisms underlying the tumor suppressor gene silencing in cancer has promoted the idea of pharmacologically relieving the inhibitory effects of DNA methylation and chromatin remodeling on gene expression. Identification of frequently methylated RCC tumor suppressor genes has highlighted potential targets for therapeutic intervention. Decitabine, the clinical form of the demethylating agent 5-aza-2′-deoxycytidine, has been used in several clinical trials, and promising responses have been reported for hematological malignancies such as myelodysplastic syndrome [170, 171]. Various studies have tested DNMT inhibitors or HDAC inhibitors either alone or in combination with conventional chemotherapeutic agents in RCC cell lines with promising results [172–174] but clinical studies are required to conclusively demonstrate the therapeutic usefulness in RCC.

4.9 Conclusions

Understanding the complexity of the epigenome and all the actors involved in modulating its interactions with genomic sequences is of fundamental importance in health and disease. Owing to the reversible and plastic nature of epigenetic alterations, these constitute an attractive target for novel therapeutic intervention. Studying epigenomic alterations and miRNAs provide opportunities for the development of innovative biomarkers to aid in disease detection, diagnosis, prognosis and prediction of response to therapy. Understanding the complex molecular mechanisms involved in epigenetics and miRNAs, may lead to more effective cancer treatments and promote the change from current cytotoxic therapies to more targeted control of malignant phenotypes.

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