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The Epigenetics of Kidney Cancer and Bladder Cancer

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Summary

This review focuses on the epigenetic alterations of aberrant promoter hypermethylation of genes, histone modifications or RNA interference in cancer cells. The current knowledge of hypermethylation of allele(s) in classical tumor suppressor genes in inherited and sporadic cancer, candidate tumor suppressor and other cancer genes is summarized gene by gene. Global and array-based studies of tumor cell hypermethylation are discussed. The importance of standardization of scoring of the methylation status of a gene is highlighted. The histone marks associated with hypermethylated genes, and the microRNAs with dysregulated expression, in kidney or bladder tumor cells are also discussed. Kidney cancer has the highest mortality rate of the genitourinary cancers. There are management issues with the high recurrence rate of superficial bladder cancer while muscle invasive bladder cancer has a poor prognosis. These clinical problems are the basis for translational application of gene hypermethylation to the diagnosis and prognosis of kidney and bladder cancer.

Keywords

RCC; bladder cancer; promoter hypermethylation; tumor suppressor gene; methylome; translational application

1. Introduction

There will be an estimated 58,000 new cases and 13,500 deaths from kidney cancer in the United States in 2010 [1]. A quarter of patients with renal cell carcinoma (RCC) present with locally advanced or metastatic disease and a third of patients who undergo resection for local disease will have a recurrence [2]. Over 90% of all kidney cancers are renal cell carcinomas (RCC) originating from the renal parenchyma. The classification of RCC comprises several histological subtypes with different genetic backgrounds and natural histories [3]. Clear cell carcinoma (75%), papillary carcinoma (10-15%) and chromophobe carcinoma (5%) account for the majority of RCC. The remaining <10% of kidney cancers are mainly transitional cell carcinomas (TCC) of the renal pelvis that, in terms of histology, biology and genetics, are similar to TCC of the bladder.

Bladder cancer is the sixth most common cancer in the western world. There will be more than 71,000 new cases of cancer in the bladder in the US this year [1]. Although up to 75-80% of new cases present as non-invasive (pathological stage Ta), stroma invasive (T1) or carcinoma *in situ* (Tis) disease, the remaining 20-25% of tumors present as muscle invasive or more advanced disease (T2-4) with a poor prognosis. Furthermore, although approximately 20% of Ta and T1 tumors are cured, after initial removal 60-70% recur at least once in 5 years and 10-20% progress to muscle invasive cancer [4]. The established

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association between tobacco, or certain occupational, exposure and bladder cancer has identified high risk populations. In the Western world, TCC represents 90% of disease. In Africa and the Middle East, squamous cell carcinoma (SCC) is the predominant cell type and is related to bilharzia infection.

Cancer is a disease initiated and driven by clonal selection of cells with either inherited (germline) or acquired (somatic) genetic or epigenetic alteration of key genes that confer a growth advantage. Oncogenes are genes whose increased or altered function can result in neoplastic transformation. To date many oncogenes have been described but only a fraction have been found to be activated in bladder or kidney cancers by somatic mutation [5-7]. Several regions of amplification have been identified by comparative genome hybridization (CGH) studies and further oncogenes likely will be discovered [8, 9]. Tumor suppressor genes are best defined as genes whose loss of function can lead to neoplastic change. Both alleles need to be inactivated by germline or somatic mutation i.e. loss of heterozygosity (LOH), point mutation, homozygous deletion or promoter hypermethylation to initiate tumor formation. Approximately twenty classical tumor suppressor genes have been identified in human cancer. In sporadic clear cell RCC, chromosome 3p deletion, and inactivation of the *VHL* tumor suppressor gene, is known to be the most common genetic alteration. In bladder cancer, inactivation of the *p53*, *Rb*, *p16*^{INK4a}/*p14ARF* and *PTEN* tumor suppressor genes occur at a moderate frequency. Other chromosomal arms have been observed to be frequently lost in RCC and bladder cancer indicating that additional tumor suppressor genes are important in tumorigenesis [3, 5].

2. DNA Methylation

Epigenetic alterations in cancer cells include DNA methylation, histone modification and RNA interference. The most studied epigenetic alteration is DNA methylation that can occur at the cytosine that precedes the guanine in a CpG dinucleotide. While CpG are generally underrepresented in the human genome sequence, the promoter regions of around half of the human genes contain a CpG-rich area termed a CgG island that are generally unmethylated in normal cells [10]. Cancer cells can show both global hypomethylation and localized hypermethylation compared to the normal cell counterpart. To date, there are few reports of hypomethylation of human oncogenes associated with activation by overexpression [11]. In part, this may be due to lack of study. Loss of imprinting (LOI) resulting in aberrant expression of the imprinted allele in cancer cells has been described in Wilms tumor and colorectal cancer [12, 13] but has not yet been clearly demonstrated in renal or bladder cancer [14]. However, it is well-established that aberrant hypermethylation of the promoter region of tumor suppressor genes is associated with transcriptional silencing and that hypermethylation is an alternative mechanism of functional inactivation [10].

2.1 Tumor Suppressor Genes Predisposing to Familial Renal or Bladder Cancer

The most frequent form of familial RCC occurs in individuals with inherited *von-Hippel Lindau* (*VHL*) syndrome. The identification of the predisposing *VHL* tumor suppressor gene located on chromosome 3p led to the finding that, in addition to inactivation by point mutation or deletion, the *VHL* gene also showed allelic inactivation by aberrant hypermethylation of the promoter region (Figure 1) that was associated with transcriptional silencing in 10-15% of familial and sporadic RCC [15]. The *VHL* tumor suppressor gene was the first gene identified as hypermethylated in RCC. *VHL* inactivation occurs only in clear cell tumors and similarly, methylation of *VHL* has been found only in clear cell RCC [15, 16]. Since *VHL* inactivation is the initiating event in familial clear cell renal tumors, it is likely an early, or even initial, event in sporadic clear cell renal tumorigenesis.

The genes that confer predisposition to other inherited forms of RCC were subsequently identified. Activating point mutations of the *MET* proto-oncogene are a cause of one form of hereditary papillary RCC [17]. No evidence of *MET* hypomethylation has been reported to date. The *Fumarate Hydratase* (*FH*) tumor suppressor gene has been identified as a predisposition gene for a second form of hereditary papillary RCC [18] and the *Birt-Hogg-Dube* (*BHD*) tumor suppressor gene for chromophobe RCC [19]. Although both these genes have typical CpG islands in the promoter region, no clear evidence of hypermethylation has been found in familial and sporadic RCC or cancers from other organ sites [16, 20, 21]. Inherited mutation of the *succinate dehydrogenase complex, subunit B, iron sulfur (Ip)* (*SDHB*) gene predisposes to early-onset RCC [22] and methylation of *SDHB* was reported in 1 of 25 sporadic RCC [23].

No common or defined familial form of bladder cancer has been identified. Familial bladder tumors when found are invariably from individuals with Hereditary Nonpolyposis Colorectal Cancer (HNPCC) due to inherited mutation of one of the *MSH2, MLH1, MSH6* or *PMS1* mismatch repair genes. TCC, predominantly of the upper tract, is the fourth commonest type of cancer in HNPCC and accounts for about 1% of bladder tumors [24]. RCC is rare in HNPCC and when found does not usually show microsatellite instability (MSI) [25]. Methylation of the *MLH1* mismatch repair gene is found in the subset of sporadic colorectal, endometrial and gastric tumors with MSI [26]. Neither sporadic bladder nor renal cancers show microsatellite instability (MSI) and *MLH1* methylation is absent or rare in renal and bladder cancer (unpublished data and ref [27]). The other mismatch repair genes do not appear to be hypermethylated in human cancer [28, 29].

Cowden syndrome is an autosomal dominant syndrome, which results in a predisposition to certain cancer types, including renal cancer [30, 31]. *PTEN*, located on chromosome 10q, has been identified as the predisposing gene for this syndrome [32]. Inactivation of *PTEN* by deletion and point mutation is evident in a minority of primary renal and bladder tumors but studies have shown no evidence of promoter methylation [33]. A *PTEN* pseudogene located on chromosome 9 can be methylated in human cells. Because of substantial sequence overlap in the promoter region with *PTEN* it is possible that some reports of *PTEN* methylation represent methylation of the psuedogene [34].

It is unclear if individuals with the disease tuberous sclerosis have a higher incidence of RCC or not. Methylation of the *TSC1* gene has not been well-examined and a solitary report found no promoter methylation of *TSC2* in hamartomas from TSC patients [35]. *TSC2* contains a typical CpG island in the promoter region but, in a preliminary study, we observed no methylation by bisulfite sequencing of 10 primary RCC and 5 RCC cell lines (unpublished data).

2.2 Classical Tumor Suppressor Genes

Allelic loss of chromosomal region 9p21 is common in most types of solid tumor. The targets of deletion are the $p16^{INK4a}$ and adjacent *p14ARF* tumor suppressor genes. Homozygous deletion is the most common mechanism of inactivation of these genes. Promoter methylation is found relatively infrequently and point mutation is extremely rare [36]. The frequency of $p16^{INK4a}$ methylation in renal cancer cell lines [37] is higher than in primary RCC [16]. This observation holds true for many genes hypermethylated in cancer. Because cell lines are invariably established from advanced tumors and can undergo clonal selection over a number of passages, the frequency of gene methylation may be unrepresentative of the primary cancer [38]. Promoter hypermethylation of $p16^{INK4a}$ is present in 5-10% of primary RCC and bladder cancer [16, 39]. In renal tumors, $p16^{INK4a}$ mutations are found in both primary and metastatic tumors from the same patient [37] and, in another study, found in all grades and stages of renal tumors [16]. In bladder cancer,

p16^{INK4a} hypermethylation appears to be more common in muscle-invasive tumors (unpublished data). The *p14ARF* gene, which shares a coding region with *p16*, has a distinct exon 1 and promoter region containing a typical CpG island [40]. The *p14ARF* promoter and exon 1 CpG island has been reported to be hypermethylated in 5-10% of primary renal and bladder tumors [16, 41].

The *Adenomatous Polyposis Coli (APC)* tumor suppressor gene is associated with both familial adenomatous polyposis (FAP) and sporadic colorectal tumors and is part of the Wnt signaling pathway. An initial study examined *APC* promoter hypermethylation in colorectal and other cancers. Hypermethylation was observed in 10% and 8% respectively of a relatively small set of bladder and renal tumors [42]. Two further studies on larger groups of bladder tumors identified *APC* methylation in 35%-45% of tumors [43, 44]. A profile in renal cancer, using a larger, more representative set of tumors, found the frequency of *APC* methylation to be 14% [16].

The *E-cadherin* (*CDH1*) gene, located on chromosome 16q22.1, has an important role in cell-cell adhesion. Inactivating point mutations of this gene have been identified to predispose to gastric cancer [45, 46] and more rarely to other epithelial tumor types. Methylation of the second allele in tumors arising in individuals with germline mutation has been reported [47]. Loss of E-cadherin function is thought to contribute to tumor progression through increased proliferation, invasion, and metastasis. *E-cadherin* expression is down-regulated in many human cancer types including bladder and kidney. Hypermethylation of *E-cadherin* was reported in 64% (9/14) of RCC lines [48]. The same study reported that hypermethylation of the promoter region of *E-cadherin* and several other tumor suppressor genes was highly, but not always, correlated with loss of expression [48]. Chung et al. found *E-cadherin* methylation in only A-498, 1 (8%) of 5 kidney and bladder lines examined [49]. In primary renal cancer, Dulaimi et al reported a methylation frequency of 11% [16]. Maruyama et al reported a methylation frequency in 36% of 98 primary bladder tumors. *E-cadherin* methylation was seen in bladder tumors of all pathological grades and stages [43, 50]. Another cadherin gene, *H-cadherin* (*CDH13*) was reported methylated in 29% of the same series of bladder tumors [43]. A detailed study across the promoter region of *E-Cadherin* revealed significant differences in levels of methylation between individual CpG sites in the same tumor cell line and between different tumor cell lines in the NCI-60 panel [51]. The same study reported that above a threshold of approximately 20% to 30% of promoter CpG sites methylated, *E-cadherin* mRNA expression was effectively silenced.

Interestingly, although around half of the twenty or so classical tumor suppressor genes identified to date can be hypermethylated in human cancer, several known to be inactivated in renal, bladder and other cancers by point mutation and deletion either lack a typical promoter CpG island, e.g. *p53*, or have a promoter CpG island that appears to be unmethylated in human cancer e.g. *PTEN* [33, 34]. The *Rb* gene, also inactivated in bladder and renal cancer, can have promoter methylation in retinoblastoma [52] but appears unmethylated in urological tumors [16]. Other genes in these tumor suppressor gene pathways are hypermethylated in genitourinary tumors i.e. *p16*^{INK4a} in the RB/p16 pathway [16, 39] and *p14ARF* in the p53/p14 pathway [16, 41].

2.3 Candidate Tumor Suppressor and Other Cancer Genes

A number of genes that are not commonly inactivated by genetic alteration, i.e. intragenic point mutation, are transcriptionally inactivated by promoter hypermethylation. Such genes have been considered candidate tumor suppressor genes. Tissue inhibitors of metalloproteinases (TIMPs) regulate extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPs). The finding of loss of expression of the *TIMP3* gene in tumor

cells led to the investigation of whether the gene was silenced by promoter methylation. *TIMP3* was reported to be methylated by Methylation Specific PCR (MSP) analysis in 78% of 36 primary renal cancer tumors across cell type, grade and stage [53]. *TIMP3* is therefore one of the most frequently methylated genes known in renal cancer. A more representative study of 100 primary renal tumors, also by conventional gel-based MSP, reported methylation in 58% of RCC [16]. However, as more quantitative technologies for the analysis of gene methylation are available, it is becoming evident that the high frequency of methylation reported for some genes includes cases where only a small proportion of the tumor cells contain methylated alleles. At present, the biological significance of such levels of methylation is unclear. Standardization of scoring a gene as hypermethylated in a tumor specimen is an important issue [54].

Another candidate tumor suppressor gene in renal cancer is the *Ras association (RalGDS/AF-6) domain family 1* gene (*RASSF1A*) [55]. *RASSF1A* is a microtubule-binding protein, which regulates mitotic progression and functions as a negative regulator of the cell cycle. *RASSF1A* is methylated in 28-91% of primary renal tumors [16, 56-58]. The differences in the percentage frequency of methylation are likely due to individual studies using primer sequences from different areas of the promoter CpG island, differences in the proportion of cell type, grade and stage of tumor, as well as the methylation analysis technology used in the study. In a large study of RCC broadly representative of cell type, grade and stage at presentation, *RASSF1A* was methylated in 45% of tumors [16]. While *RASSF1A* methylation has been identified in both clear cell and papillary RCCs, two studies reported that the frequency of methylation is higher in papillary compared to clear cell [16, 57]. *RASSF1A* methylation is also found in chromophobe tumors [16] and it is the most frequently methylated gene in early stage (organ-confined) RCC. *RASSF1A* is frequently methylated (35-60%) and an early event in bladder cancer [43, 59, 60]. *RASSF1A* expression is either lost or reduced in concordance with promoter methylation [55].

One of the first genes found to be methylated in genitourinary cancer was the carcinogen detoxification enzyme *glutathione S-transferase pi-1 (GSTP1)* gene. Hypermethylation of the promoter region of the *GSTP1* gene is found in the majority (70-90%) of primary prostate carcinomas, but not in normal prostatic tissue or in benign hyperplasia of the prostate [61]. *GSTP1* has also been identified as methylated in a minority (<10%) of bladder and renal cancers representative of cell type, grade and stage at presentation [16, 62, 63].

O^6-Methylguanine-DNA Methyltransferase (MGMT), a DNA repair gene, has aberrant promoter methylation associated with loss of expression in several cancer types [64]. *MGMT* promoter hypermethylation is relatively infrequent in both renal (6-8%) and bladder (2-5%) tumors [16, 43, 62, 65].

The *death-associated protein kinase* (*DAPK1*) gene is located on chromosome 9q34.1, an area of frequent LOH in bladder cancers [66]. The cellular activities of *DAPK1* are critical for antagonizing caspase-dependent apoptosis to promote cell survival under normal cell growth conditions. Kissil et al. found that several cancer cell lines, including bladder and renal, lack *DAPK1* mRNA and protein expression. Reactivation of *DAPK1* expression following azacytidine treatment was also observed. Methylation was found in 4 of 14 (29%) bladder cancer cell lines and in 2 of 5 (40%) RCC cell lines [66]. Further studies by Katzenellenbogen et al. found a correlation between the loss of *DAPK1* expression and promoter hypermethylation [67]. However, most studies report that *DAPK1* methylation is uncommon in primary RCC [68, 69] and primary bladder tumors [27, 43]. In contrast, Tada et al. reported that overall, 29% of bladder tumors showed *DAPK1* methylation. *DAPK1* methylation was identified as a marker of recurrence in stage Ta and T1 bladder cancer. 88% of papillary bladder tumors with *DAPK1* methylation recurred within 15 months, while

71% of tumors that are not methylated for *DAPK1* had not recurred within 24 months [70]. Another study used qMSP and reported 100% of bladder tumors and 100% of primary RCC to show methylation of *DAPK1* albeit of very heterogeneous levels [71]. The apparently conflicting published data on frequency of methylation of DAP-Kinase highlights again the need for standardization of assay and scoring.

Studies have reported 15-19% of bladder tumors to show hypermethylation of the *retinoic acid receptor 2* (*RARβ2*) gene [43, 44, 72]. Methylation of *RARβ2* in renal tumors was comparable, with a frequency of 12% [16]. This gene encodes retinoic acid receptor beta, a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators. This receptor localizes to the cytoplasm and to subnuclear compartments. It binds retinoic acid, the biologically active form of vitamin A that mediates cellular signaling in embryonic morphogenesis, cell growth and differentiation. The RARβ2 protein is thought to limit growth of many cell types by regulation of transcription.

Laminin-5 (LN5), a glycoprotein that is secreted by epithelial cells, is composed of α3, β3 and γ2 chains encoded by the three LN5 genes: *LAMA3*, *LAMB3*, and *LAMC2* respectively. The frequency of methylation of the LN5 genes in bladder tumors is reported to range from 21-45%. *LAMA3*, the most frequently methylated of the three genes, had a frequency of 45% in bladder tumors. *LAMB3* was methylated in 25% and *LAMC2* in 23% of the same subset of tumors. It was reported that patients with *LAMC2* methylation had a shorter survival than patients that did not have methylation. [73]

Reprimo, a gene involved in the p53-mediated cell cycle arrest at the G2/M checkpoint, was reported to be frequently hypermethylated with associated loss of gene expression in a number of varying cancers (>30%). The methylation frequency in bladder cancer was lower (19%) [74]. The methylation status of *Reprimo* in renal cancer has not been studied.

Promoter hypermethylation of the *fragile histidine triad* gene (*FHIT*) was identified in 11-16% of bladder tumors and was correlated with a poor survival rate in patients [43, 75]. *FHIT* hypermethylation has also been reported in 54% of clear cell RCC [76]. This gene, a member of the histidine triad gene family, encodes a protein involved in purine metabolism. The gene encompasses the common fragile site FRA3B on chromosome 3p, where carcinogen-induced damage can lead to translocations and aberrant transcripts of this gene.

HAI-2/SPINT2 encodes Kunitz-type protease inhibitor, which functions as a regulator of hepatocyte growth factor (HGF) activity. Tumor suppressor activity as well as inactivation by hypermethylation of *SPINT2* has been identified in both the clear cell (30%) and papillary (40%) subtypes of RCC. [77]

Another candidate TSG, *BLU* also known as *ZMYND10* for zinc finger, MYND-type containing 10, has been identified on the short arm of chromosome 3, located upstream of the *RASSF1A* gene. *BLU* was reported to have a methylation frequency of 50% in kidney cell lines. It was noted that, although *RASSF1A* and *BLU* are in close proximity to one another, no correlation was found between promoter methylation of these two genes. [78]

The *secreted frizzled receptor protein* (*SFRP*) family, involved in the Wnt signaling pathway, has been studied in both kidney and bladder cancer. Promoter hypermethylation of *SFRP1* has been reported to be 29% in papillary bladder cancer [79]. In a large set of bladder tumors, the frequency of methylation was *SFRP1* (18%), *SFRP2* (*52%*), *SFRP4* (*9%*) and *SFRP5* (*37%)* [80]. In RCC, hypermethylation of *SFRP1* was reported in 68% of 38 RCC and correlated with loss of expression by immunohistochemical analysis [81] and hypermethylation of *SFRP2* correlated with loss of expression in RCC cell lines [82]. A study of *SFRP1*, *2*, *4* and *5* and other Wnt antagonist genes *DKK3* and *Wif1* found each gene

to be methylated in around 50% and *Wif-1* in 73% of RCC [83]. Standardized technology and scoring will be necessary to determine the true frequency of biologically relevant levels of methylation of *SFRP* and other genes.

The *transforming growth factor β* (*TGFβ*) family of genes regulates a variety of cellular functions. Regulation of the *TGFβ* genes has been identified in cancer cells during different stages of pathogenesis [84-86]. Suzuki et al. examined the methylation status of three TGFβ-related genes; *DRM/Gremlin* a member of the bone morphogenic protein antagonist family implicated in cellular hypertrophy, the transcription factor *RUNX3*, and *HPP1/TMEFF2* transmembrane protein with EGF-like and two follistatin domains in human cancers with various clinicopathologic features. [87] In bladder cancer, *DRM/Gremlin* was reported as methylated in 51% of tumors examined, while *HPP1* has a methylation frequency of 35% and *RUNX3* showed methylation in 42% of tumors. In all three genes, methylation was tumor-specific [87].

The *Human homeo-box B13* (*HOXB13*) gene has been identified as methylated in 30% of RCC. Loss of expression of *HOXB13* gene in primary RCC and cell lines correlated with methylation status. [88]

DAL-1/4.1B, an actin binding protein, was found to be methylated in 47% of 19 renal cancer cell lines and 45% of 55 clear cell renal tumors [89].

The promoter region o*f ABCG2*, an ATP-binding cassette transmembrane protein implicated in clinical drug resistance, has been reported to be densely methylated in RCC cell lines and to show reactivation of expression after azacytidine treatment [90].

The location of an imprinted gene *DLK1* in the chromosomal region 14q, commonly deleted in RCC, prompted a report that *DLK1* and *GTL2* are reciprocally imprinted genes in the manner of *IGF2/H19* since methylation status of *GTL2* correlated with expression of *DLK1* in RCC lines [91].

The apoptosis-associated interferon response gene *XAF1* is methylated and reactivated by azacytidine treatment in the ACHN renal tumor cell line [92]. Methylation associated with down-regulation of expression has been reported in 6 of 7 (86%) kidney and 16 of 18 (89%) bladder primary tumors by conventional MSP [93]. However, qMSP analysis of primary RCC detected methylation in 10% of 91 cases, suggesting other mechanisms for transcriptional downregulation of *XAF1* [94].

2.4 Global Gene Hypermethylation Studies

To date, the majority of genes known to undergo aberrant methylation in cancer cells have been identified by a candidate approach. By definition, this has resulted in the examination of a limited number of genes. Recently, a global profile of genes silenced by hypermethylation in RCC was generated by an expression microarray-based analysis of genes reactivated in 4 RCC lines after treatment with the demethylating drug 5Aza-2 deoxycytidine (5Aza-dC) and histone deacetylation inhibiting drug trichostatin A (TSA) [95]. Between 111 to 170 genes were found to have at least 3-fold upregulation of expression after treatment in each cell line. To establish the specificity of the screen for identification of genes epigenetically silenced in cancer cells, a subset of 12 upregulated genes was validated. The promoter methylation status and transcription status of the 12 genes were validated by semi-quantitative RT-PCR of untreated and treated cell line cDNA and by bisulfite sequencing and methylation specific PCR (MSP) of tumor and normal cell DNA. Three of the 12 genes (*IGFBP1, IGFBP3* and *COL1A1*) showed promoter methylation in tumor DNA but were unmethylated in normal cell DNA, 1 gene (*GDF15*)

was methylated in normal cells but more densely methylated in tumor cells, and 1 gene (*PLAU*) showed cancer cell specific methylation that did not correlate well with expression status. The remaining seven genes had unmethylated promoters. However, there is evidence for at least one of these genes (*TGM2*) to be regulated by another gene, *RASSF1A* [96], which was methylated in the RCC lines. It is likely that the epigenetic reactivation of particular genes leads to a cascade of upregulation in diverse pathways and networks. Other genes may be upregulated as a direct response to the stress of 5Aza-dC treatment.

Conventional MSP analysis of 32 primary, mainly organ-confined (stage I or II), renal tumors of the most common histological cell types (20 clear cell, 10 papillary and 2 chromophobe) was performed. *IGFBP1* was methylated in 31%, *IGFBP3* in 37%, and *COL1A1* in 56% of the primary RCC. Because conventional MSP is not quantitative, we cannot be certain the methylation of the genes in the primary renal tumors is clonal, as we observed in the RCC cell lines, without further studies. The frequent methylation of these 3 genes in early stage tumors of the most common histological subtypes of RCC implicates these genes in renal tumorigenesis

In regard to the putative role of these genes in cancer, the insulin like growth factor binding proteins 1 (*IGFBP1*) and 3 (*IGFBP3*) are major forms of the IGF-binding protein family that can inhibit the growth promoting activity of both IGF I and IGF II. IGFBP3 is known to inhibit cell growth by sequestering IGF I, however, the mechanism by which IGFBP1 exerts its activity is less well understood. Clearly, methylation-based silencing of *IGFBP1* and *IGFBP3* could provide growth advantages to the neoplastic cell. Activation of this pathway may be of therapeutic advantage in limiting tumor growth.

COL1A1 is the human gene coding for the α1 chain of type I collagen, the major extracellular matrix component of skin and bone. Changes in the synthesis of type I collagen are associated with normal growth and tissue repair processes. Alterations in extracellular matrix composition have been implicated in tumor progression and metastasis. The *COL1A2* gene has been reported as hypermethylated in bladder cancer [97]. Both the *IGFBP* and the *COL1A* gene families appear prone to hypermethylation and it is interesting that other global epigenetic screens have shown reactivation of gene families e.g. *IFN* in bladder [98] and *SFRP* family members in colorectal cancer [99]. Another epigenetic reactivation study reported hypermethylation of *IGFBP3* and *PLAU* as well as newly identified hypermethylation of *KRT19* and *CXCL16* in RCC [100]. A more extensive study of 11 RCC cell lines by the same group identified eight genes (*BNC1, PDLIM4, RPRM, CST6, SFRP1, GREM1, COL14A1* and *COL15A1*) that were frequently (>30%) methylated in primary RCC [101]. A global reactivation study of the ACHN renal tumor cell line identified the *Ubiquitin carboxyl-terminal esterase L1* (*UCHL1*) gene, also called *PGP9.5*, as hypermethylated in RCC. The same study also reported upregulation of *COL1A1* in ACHN cells after demethylating drug treatment [82]. Another, independent, study reported *UCHL1* as hypermethylated in around a third of primary RCC [102]. A mass spectrometry analysis of a set of genes down-regulated in RCC compared to normal renal tissue in RCC identified *SCNN1B, SYT6, DACH1* and *TFAP2A* as hypermethylated in RCC [103]. A first generation methylation array analysis of RCC identified many genes not previously reported to be methylated in RCC including *TWIST* and *SFRP3* [104].

Global epigenetic screens of several bladder cancer cell lines identified the *lysyl oxidase-like 1* and *4* (*LOXL1* and *LOXL4*) genes as frequently hypermethylated in primary bladder cancer [105] and *FGF18* and *MMP11* as hypermethylated in the untreated cell lines [106].

2.5 Differing Frequencies of Methylation of a Gene in the Literature

There are several likely explanations for the discrepancies in the reported frequency of methylation of a gene between different studies. These include differences in the characteristics of the tumor set, i.e. number studied, cell type, grade, stage, and percentage tumor cell content of biopsy [107]. The frequency of methylation of a gene in tumor cell lines does not always correspond with the frequency in a representative set of primary tumors [38]. The efficiency of the bisulfite modification and the technology used for analysis is relevant since cases where only a small proportion of the tumor cells (5-10%) contain methylated alleles could be missed by direct bisulfite sequencing or pyrosequencing for example. Conventional gel-based MSP cannot readily distinguish between a tumor with clonal (in 100% of cells) methylation and a tumor with methylation in only 1% of cells. As previously mentioned, the biological significance of low levels of methylation is unclear. Primer design and location can differ between studies and heterogeneity in the methylation of individual CpG sites between different tumors can lead to MSP, which interrogates only a few CpG sites, scoring the same tumor specimen as methylated or unmethylated depending upon which particular CpG sites the primers are directed to. The stringency of a MSP reaction depends on both the salt concentration in the PCR buffer and the annealing temperature which can vary between laboratories [54].

These points again raise the question of how methylation should be scored. The increasing use of quantitative real time PCR for methylation analysis provides more information than conventional MSP but there is still no standardization for reporting of results. A direct sequence readout that is quantitative remains the gold standard [54]. Direct bisulfite sequencing which is only semi-quantitative, provides a long sequence read, and is not subject to cloning bias or direct pyrosequencing, which is more quantitative but gives a short read, are at present the closest to that goal. Current methylation arrays also have issues including that only a small minority of CpG sites of a gene promoter are represented. Additional issues are that some array-based studies do not independently validate the methylation by a different technology and that some gene methylation publications do not show any primary data.

There is also the question of whether only methylation of functional significance i.e. at or above the threshold (by extent and/or position within a given gene promoter) for loss of expression be scored? This will likely vary from one gene to another. Reexpression of a methylated gene after demethylating drug treatment of cultured cells is only an indication that the methylation of that particular gene is of functional significance as it is possible that demethylation of a transcription factor or upstream regulatory gene has restored expression. Even if methylation of a gene can be shown to have functional significance that does not imply a causal role in tumor initiation or progression. If a CpG island methylator phenotype (CIMP) [108] is clearly demonstrated in bladder cancer or RCC it will complicate the identification between driver and passenger gene hypermethylation in the same way that has occurred with small insertions or deletions inactivating genes in mismatch repair deficient tumors [109].

3. Histone Modifications

The crosstalk between DNA methylation, histone deacetylation and the chromatin state reinforces the expression status of a gene promoter. Particular patterns of histone marks are found at hypermethylated gene promoters in cancer cells [110]. The nucleosome is a subunit of chromatin that comprises a short length of 146bp of DNA wrapped around a core of histone proteins consisting of two subunits each of H2A, H2B, H3 and H4 forming an octamer. Histone core subunits share a common structure including an extended tail that is the site of post-translation modifications. The most common modifications to histone tails

include acetylation, methylation, phosphorylation and ubiquitylation. These modifications have the ability to enhance or block transcription factor binding and thereby initiation of transcription. Profiles of the many potential histone modifications in cancer cells are only beginning. Histone H3-lysine 9 methylation has been associated with aberrant gene silencing in the T24 bladder tumor cell line [111]. Modifications of histone H4 have been reported in several tumor types although bladder or renal tumors were not examined [112]. A study of characteristic patterns of expression of selected histone modifier genes reported that *EZH2* gene expression distinguished renal tumor from paired normal renal tissue. The pattern of expression over all 12 genes studied could discriminate bladder tumor from normal bladder. Tissue-specific patterns of expression across the 12 genes was also evident [113]. A comparison of array CGH and transcriptome analysis in bladder carcinomas identified chromosomal regions with down-regulation of expression but no loss of copy number and so yielded an overview of regional epigenetic alteration. One such copy number-independent region was validated as a region of epigenetic alteration in that loss of expression was due to tumor-specific aberrant histone methylation in the absence of DNA methylation [114]. Lower global levels of histone H3 lysine 4 dimethylation (H3K4me2) and H3K18 acetylation, and to a lesser extent H3K9me2, are reported to predict poorer prognosis in kidney cancer patients [115]. A study of histone H3 lysine 4 mono-methyl (H3K4me1), -di-methyl (H3K4me2) and -trimethyl (H3K4me3) levels on a tissue microarray of 193 RCC reported an inverse correlation with tumor grade and stage and patient survival [116].

4. MicroRNAs

MicroRNAs (miRNAs) are short (22 nucleotide) noncoding RNAs that base pair 2-8 nucleotides of their sequence to the 3’-UTR of complementary mRNA transcripts and facilitate target mRNA degradation. A single miRNA can pair to and post-transcriptionally regulate the expression of many mRNAs. Several hundred miRNAs have been identified in the human cell. The availability of array technology has led to profiles of differences in miRNA expression levels between; normal and cancer cells, grade and stage of a cancer, histological cell types, and prognostic subgroups. MiRNAs have been shown to growth-promoting or growth-inhibitory. An early study examined the relationship between the chromosomal location of miRNAs and alterations in copy number and reported that more miRNAs were located in areas deleted, rather than amplified, in human bladder cancer cells [117]. An early study profiled miRNA expression in the T24 bladder cancer cell line that showed >3-fold upregulation of 17 of 313 human miRNAs after treatment with 5Aza-2 deoxycytidine (5Aza-dC). One of the upregulated miRNAs, miR-127, is expressed in normal cells but not in tumor cells, is embedded in a CpG island and highly induced by its own promoter. This suggests it is epigenetically silenced in cancer cells and may have a tumor suppressor function [118]. Several profiles of miRNA expression in normal cells compared to renal or bladder cancer and also by tumor stage and patient outcome have been reported. Friedman et al examined pooled 9 TCCs and a pool of the matched normal transitional cells to identify a signature of miRNA expression in TCC. miR-101 tumor suppressor by down-regulation of EZH2 and consequent genome-wide effects on chromatin state [119]. Two independent studies have reported up-regulation of miR-21 or down-regulation of miR-145 in bladder cancer compared to normal urothelium [119-121]. A recent study describes miR-200c expression as a marker of progression in bladder cancer [122]. In RCC, more than one independent study has reported downregulation of miR-141 and miR-200c [123, 124] and upregulation of miR-210 [123, 125] in clear cell RCC compared to normal renal parenchymal tissue. MiR-141 and miR-200c can inhibit Epithelial to Mesenchymal Transition (EMT) by directly targeting ZEB1 and SIP1, which are repressors of E-cadherin [126]. As the field emerges, the impact of differences in source and preparation of normal cells or tumor cells, array and next generation sequencing platforms,

statistical analysis, and extent of validation on the published findings to date will become clearer.

5. Translational Applications of Gene Methylation

5.1 Diagnosis and Prognosis

Aberrant methylation of cancer genes has been found in different histological cell types and all pathologic grades and stages of genitourinary cancer across patients of both sexes and of all ages and ethnicities [107]. The natural history of sporadic renal cancer is unclear but the finding of hypermethylation in kidney tumors of the lowest pathological stage (T1a) and grade (I), including tumors as small as 2cm in size, indicates that methylation can be a relatively early event in renal tumorigenesis [16]. Similarly, gene methylation is present in grade I, stage Ta tumors and carcinoma *in situ* (CIS) of the bladder [39, 62]. In general, classical tumor suppressor genes and some candidate tumor suppressor genes have been found to be unmethylated in normal transitional cells and normal renal cells although age-related gene methylation will be an increasingly important focus of study [107].

Because tumor suppressor and other cancer gene hypermethylation is a common, early and cancer specific alteration as well as amenable to detection by the sensitive MSP technique capable of detecting one methylated allele from a neoplastic cell in a background of several thousand unmethylated alleles from normal cells, a number of feasibility studies of methylation-based detection of cancer in body fluids were performed [107]. Using conventional MSP, an identical pattern of gene hypermethylation to that in the RCC was detected in 44 of 50 (88%) matched urine DNA. Gene methylation was positively detected in pre-operative urine from patients with organ-confined (stage I and II) RCC, including tumors as small as 2.2 cm in size. In contrast, methylation was absent from normal renal tissue and urine obtained from normal and non-neoplastic disease controls [127]. Other investigators confirmed these findings in a subsequent quantitative real time MSP study of gene methylation in RCC patient urine DNA [65]. Several studies have used panels of genes methylated in bladder cancer, commonly including the *RASSF1A, p16, p14ARF, DAPK1, APC* genes as well as *laminin-5*, apoptosis and Wnt-antagonist family genes, to demonstrate sensitive and specific detection of gene methylation in the paired pre-operative urine [39, 62, 73, 83, 128-130]. A recent study found the *TWIST1* and *NID2* genes to be frequently hypermethylated in bladder tumors and by qMSP of urine sediment DNA from several hundred patients with bladder cancer detected methylation of these genes with 90% sensitivity and 93% specificity [131].

One barrier to translational application of gene methylation for early detection is that the vast majority of genes identified to date can be methylated in all the genitourinary cancer types as well as cancers in other organ sites. Relatively few genes have been identified to have organ specific or cell type specific methylation that would facilitate differential diagnosis. *VHL* methylation is restricted to clear cell RCC. Methylation of *Timp-3* is more common in RCC than in some other tumor types although this has not been well studied [53]. The *RASSF1A* and *SPINT2* genes are more frequently methylated in papillary than clear cell RCC [16, 57, 77, 104, 132] while *IGFBP1* and *COL1A1* gene methylation is more common in clear cell RCC [95]. *CDH1* methylation was reported significantly higher in clear cell RCC compared to chromophobe RCC or oncocytoma [132]. The <10% of bladder cancer that is non-transitional cell has not been extensively profiled for gene methylation. One study reported that *RASSF1A, APC, p16, DAPK1* and *RARβ2* are also hypermethylated in SCC of the bladder [133]. More genes methylated at different frequencies in different cell types will likely be identified [95].

Gene methylation may disrupt critical pathways, and thus likely plays an important role in the progression of renal and bladder tumorigenesis. The potential association of methylation status of specific genes with the biology of the tumor may facilitate prognostic classification in terms of response to targeted therapy and disease outcome and therefore merits study. For example, the γ-catenin gene has been associated with poor prognosis in RCC patients [134] and methylation of particular genes associated with progression risk in bladder cancer [135].

5.2 Epigenetic Therapy

Because demethylating drugs such as azacytidine and histone deacetylase (HDAC) inhibitors such as suberoylanilide hydroxamic acid (SAHA) can reactivate expression of epigenetically silenced genes there is much interest in the therapeutic potential of these agents and some early studies. A Phase I trial of low dose 5-Aza-dC and high dose interleukin-2 (IL-2) in 5 RCC patients reported a temporal overlap between decitabine-induced DNA hypomethylation and re-expression of methylated genes, and immune activation by high-dose IL-2. The study demonstrated this combination could be safely administered although no antitumor activity was noted in the small number of RCC patients [136]. A phase II trial of the DNMT inhibitor MG98 in metastatic RCC patients is underway [137]. Another phase II study is of the VEGF-inhibitor bevacizumab and the HDAC inhibitor vorinostat in metastatic RCC patients [138]. Combination therapy of an mTOR inhibitor and an HDAC inhibitor reduced HIF1a expression and showed growth inhibitory effects on a VHL-deficient RCC cell line greater than with single agents [139]. Treatment with azacytidine and cisplatin showed synergistic growth suppression in five bladder cancer cell lines [140]. Another bladder cancer cell line-based study reported upregulation of 17 of 313 human miRNAs after treatment with 5Aza-dC [118]

6. Future Perspective

The study of cancer epigenetics is still in a formative period. DNA methylation is the best studied of the types of epigenetic alteration present in the cancer cell. The number of genes with aberrant methylation in the human cancer cell is not known but a reasonable estimate might be that 1%, or 250 genes, of the human genome can be aberrantly methylated in a tumor cell [141, 142]. Approximately 50 genes are discussed in this chapter. Subsequent examination of further individual cancer genes, as well as array-based discovery [143] and high-throughput-based global profiles [104] of gene methylation, in larger numbers of specific genitourinary tumor types will almost certainly reveal more classical tumor suppressor genes and other important cancer genes to be methylated in kidney and bladder cancer. Studies of histone modifications, chromatin remodeling and microRNAs in cancer are emerging. Such studies combined with the methylome and transcriptome should ultimately lead to an integrated epigenome of kidney and bladder cancer. Changes in the epigenome of the normal progenitor cells of kidney and bladder cancer through ageing [144] and environmental influences [145] and a better understanding of the earliest steps in the development of kidney and bladder cancer lesions will be other major areas of study.

Executive Summary

Introduction

Epigenetic alterations are evident in bladder and renal tumor cells compared with the normal progenitor cell.

DNA hypermethylation of CpG islands in the promoter regions of genes is well-studied. Initial studies of histone modification or miRNA expression report differences between tumor and normal cells.

Tumor Suppressor Genes Predisposing to Familial Renal or Bladder Cancer

The promoter region of the VHL gene is hypermethylated in a subset of clear cell RCC

Classical Tumor Suppressor Genes

Why some genes with a promoter CpG island e.g. *Pten* are unmethylated in human tumor cells and whether other epigenetic alterations silence transcription of these genes remains unclear.

Candidate Tumor Suppressor and Other Cancer Genes

Clonal selection suggests that hypermethylation of cancer genes confers a growth advantage but the driver versus passenger question is pertinent.

Global Gene Hypermethylation Studies

Azacytidine reactivation coupled with expression array analysis as well as first generation gene methylation array studies have produced large sets of genes, however only a small subset have been validated. Consortium initiatives such as TCGA will be a source of potential biomarkers.

Differing Frequencies of Methylation of a Gene in the Literature

For standardization, a consensus is needed on the definition of whether an individual gene in a particular tumor is ‘methylated” or not based on normal and tumor, extent and position of methylation of CpG sites, effect on transcription and whether a minor or major clone within the tumor.

Translational Applications of Gene Methylation

Currently, there is evidence for a limited number of methylated genes to have clinical utility for early detection, prognostic and predictive classification of response in cancer. Better designed validation studies are required.

Combinations of epigenetic drugs with a standard therapy are beginning to be investigated.

Conclusions

Elucidation of the DNA methylome in cancer appears possible.

An integrated methylome, mRNA and miRNA transcriptome of kidney and bladder cancer will be produced by the TCGA.

The role of epigenetic alterations arising from environmental interaction and ageing in the risk of developing cancer will be an important area of study.

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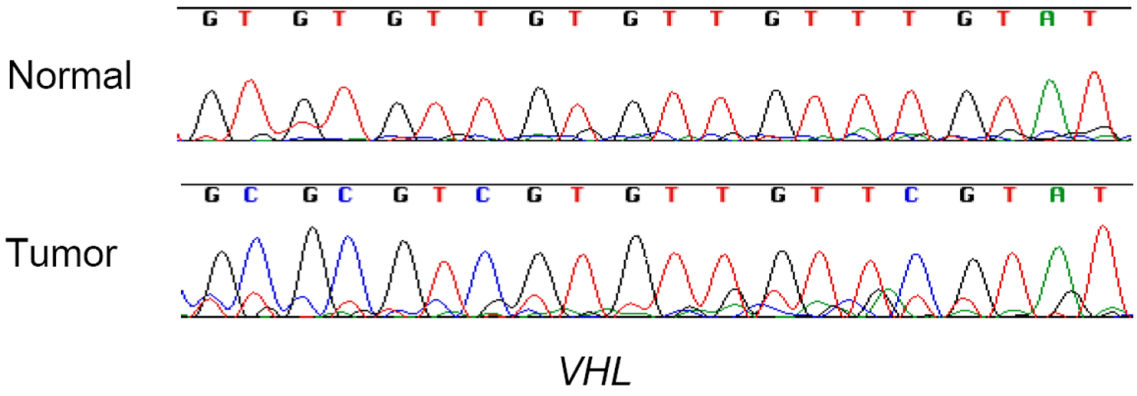

Figure 1.
Direct bisulfite sequencing of the promoter region of the VHL gene in normal renal cells and a clear cell renal tumor. The presence of cytosine (C) in the tumor DNA sequence indicates methylation.

Table 1

Selected classical tumor suppressor genes identified as hypermethylated in cancer. Function is from Gene Ontology. TSG = classical tumor suppressor gene. Methylation frequency refers to primary tumors unless stated. ND = Not done. Invariably, bisulfite sequencing was used as an initial assay followed by MSP of a larger number of tumors. COBRA is also used by some researchers.

Gene Name	Chromosomal Location	Function	Methylation Frequency		References
			Kidney	Bladder	
VHL	3p25.3	TSG, transcription factor binding	10-15%	ND	[15, 16]
BHD	17p11.2	TSG, cell cycle	None	ND	[20, 21]
FH	1q43	TSG, cell cycle	None	ND	[16]
SDHB	1p36.1	Mitochondrion respiratory chain	4%	ND	[23]
MLH1	3p21.3	TSG, DNA mismatch repair	None	None	[27, 68]
PTEN	10q2.31	TSG, Regulation of AKT1 signaling pathway	None	None	[33]
$p16^{INK4a}$	9p21.3	TSG, Cell cycle regulation	5-10%	5-10%	[16, 27, 39, 43]
$p14^{ARF}$	9p21.3	TSG, Cell cycle regulation	5-10%	5-10%	[16, 27, 41]
APC	5q22.2	TSG, Wnt receptor signaling pathway	8-14%	10-45%	[16, 42-44]

Table 2

Selected candidate tumor suppressor and cancer genes identified as hypermethylated in cancer. Function is from Gene Ontology. Methylation frequency refers to primary tumors unless stated. ND = Not done. Invariably, bisulfite sequencing was used as an initial assay followed by MSP of a larger number of tumors. COBRA is also used by some researchers.

Gene Name	Chromosomal Location	Function	Methylation Frequency		References
			Kidney	Bladder	
E-cadherin	16q22.1	TSG, Cell-cell adhesion	11%	36%	[16, 43]
H-cadherin	16q23.3	Cell adhesion protein; negative regulator of neural cell growth	ND	29%	[43]
Timp-3	22q12.3	Inhibitor of proteolytic matrix metalloproteinase activity	58-78%	ND	[16, 53]
RASSF1A	3p21.31	Negative cell cycle regulation	28-91%	35-60%	[16, 39, 43, 56-58, 60]
GSTP1	11q13.2	Detoxification	<10%	<10%	[16, 27, 63, 68]
MGMT	10q26.3	DNA repair	6-8%	2-5%	[16, 43, 64]
DAP-Kinase	9q34.1	Positive regulator of apoptosis	None	4-9%	[27, 41, 43, 68]
RARβ2	3p24.2	Transcriptional regulation	12%	15-19%	[16, 43, 44, 72]
LAMA3	18q11.2	Cell adhesion, Signal transduction	ND	45%	[73]
LAMB3	1q32.2	Mediator of cellular attachment, migration and organization	ND	25%	
LAMC2	1q25.3	Mediator of cellular attachment, migration and organization	ND	23%	
Reprimo	2q23.3	Cell cycle control	ND	19%	[74]
FHIT	3p14.2	Cell cycle	ND	11-16%	[43, 75]
SPINT2	19q13.2	Inhibitor of HGF activator	33%	ND	[77]
BLU	3p21.3	Unknown	50% cell lines	ND	[78]
SFRP1	8p11.1	Modulator of Wnt signaling.	47-68%	18-29%	[79-81, 83]
SFRP2	4q31.3	Modulator of Wnt signaling.	53%	52%	[80, 83]
SFRP3	2q32.1	Modulator of Wnt signaling.	>30%	ND	[104]
SFRP4	7p14.1	Modulator of Wnt signaling.	53%	9%	[80, 83]
SFRP5	10q24.1	Modulator of Wnt signaling.	56%	37%	[80, 83]
DRM/Gremlin	15q13.3	Inbibits TGFb signaling	>30%	51%	[87, 101]
RUNX3	1p36.11	Facilitates TGFb signaling	ND	35%	[87]
HPP1	2q32.3	Inbibits TGFb Signaling	ND	42%	[87]
HOXB13	17q21.32	Vertebrate Development	30%	ND	[88]

Gene Name	Chromosomal Location	Function	Methylation Frequency		References
			Kidney	Bladder	
DAL-1/4.1B	18p11.31	Actin Binding Protein	45%	ND	[89]
ABCG2	4q22.1	Xenobiotic transporter	67% cell lines	ND	[90]
XAF1	17p13.1	Zinc ion binding	10-86%	89%	[93, 94]
IGFBP1	7p13	IGF binding protein	31%	ND	[95]
IGFBP3	7p13	IGF binding protein, inhibitor of cell growth	37%	ND	[95]
COL1A1	17q21.33	Extracellular matrix component; growth and tissue repair	56%	ND	[95]
COLlA2	7q21.3	Extracellular matrix component; growth and tissue repair	ND	63%	[97]
KRT19	17q21.2	Cytoskeleton structure	38%	ND	[100]
CXCL16	17p13.2	Transmembrane chemokine	42%	ND	[100]
UCHL1/PGP9.5	4p13	Processing of ubiquitin precursors and of ubiquitinated proteins	37-41%	ND	[82, 102]
LOXL1	15q24.1	Extracellular matrix	ND	70%	[105]
LOXL4	10q24.2	Extracellular matrix	ND	40%	[105]
TWIST	7p21.1	Transcription factor, negative regulation of cellular determination	>20%	>70%	[104, 131]
NID2	14q22.1	Cell adhesion, extracellular matrix interaction	ND	>70%	[131]
PDLIM4	5q31.1	Protein binding	>30%	ND	[101]