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Epigenetic Biomarkers in Bladder Cancer

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1. Introduction

1.1 Epigenetics and cancer: An overview

Genetic and epigenetic alterations are hallmarks of human cancer. In the last few decades, it has been well established that epigenetic changes are important events in human cancer development and progression in addition to genetic alterations (such as chromosomal rearrangements, aneuploidies and point mutations). Epigenetics refers to the study of changes in gene expression that are determined by mechanisms other than changes in the DNA sequence. Epigenetic phenomena include X-chromosome inactivation, genomic imprinting, cellular differentiation and the maintenance of cell identity. These events are mediated by several molecular mechanisms, including DNA methylation, post-translational histone modifications and various RNA-mediated processes. Many studies in the field of epigenetics have focused on the effects of histone modifications and DNA methylation in the transcription process because these mechanisms are often linked and interdependent (Ballestar, 2011). A variety of methods are currently being applied to detect epigenetic changes, and the past two decades have shown an exponential increase in novel approaches aimed at elucidating the molecular basis of epigenetic inheritance.

DNA methylation is the most well studied epigenetic modification in human diseases (Fernandez et al., 2011). It involves the addition of a methyl group to the 5 carbon of a cytosine that is immediately followed by one guanine; i.e., DNA methylation typically occurs in a CpG dinucleotide context. CpG dinucleotides are generally underrepresented in the genome due to the increased mutation frequencies of the methylcytosines that are spontaneously converted to thymines. However, within the regions that are known as CpG islands, these dinucleotides are found at higher frequencies than is expected. It is believed that the human genome is comprised of approximately 38,000 CpG islands, and a large proportion of them (~37%) are located in the 5′ gene regulatory regions (promoters). The aberrant content of DNA methylation (global genome hypomethylation) and patterns of cytosine methylation, especially hypermethylation in promoter-associated CpG islands, are known to be associated with cancer. Gene-specific promoter hypermethylation causes the breakdown of normal cell physiology by silencing tumor suppressor genes, while DNA hypomethylation can reactivate oncogenes and repetitive sequences of the genome and lead to chromosomal instability (Sawan et al., 2008).

Histones (H2A, H2B, H3 and H4) are the main protein components of chromatin that package and order DNA into structural units that are called nucleosomes. The histone code consists of post-translational covalent changes of specific amino acid residues that are located at histone tails (NH_2 terminal regions). These modifications include methylation, acetylation, phosphorylation, poly-ADP ribosylation, ubiquitinylation, sumoylation, carbonylation and glycosylation (Kouzarides, 2007). The histone code and DNA methylation interact to promote the regulation of specific gene activity and mediate chromatin accessibility and compaction by changing the local chromatin structure, as has been reported to occur during the silencing of tumor suppressor genes. In cancer cells, the hypermethylation of CpG islands in the promoter regions of tumor suppressor genes was associated with a specific profile of histone markers such as the loss of acetylation of histones H3 and H4, loss of H3K4 trimethylation, and gains of methylation in lysine residues of histone H3 (such as H3K9 and H3K27) (Portela & Esteller, 2010).

The most recently discovered epigenetic modification is mediated by a small class of RNAs that are also known as microRNAs (miRNAs). These molecules promote the silencing of target genes by associating with the 3′ untranslated region of messenger RNA (mRNA), which culminates in endonucleolytic cleavage, mRNA degradation by deadenylation or the inhibition of mRNA translation (Valeri et al., 2009). It is estimated that at least 30% of all human genes are regulated by miRNAs. Similar to the protein-coding genes, the down-regulation of miRNAs in cancer cells has been correlated with the presence of DNA hypermethylation in the regulatory regions. In addition, these molecules (named epi-miRNAs) were recently found to regulate epigenetic enzymes, such as DNA methyltransferases and histone deacetylases. Thus, it is possible that epi-miRNAs could indirectly affect the expression of cancer-related genes (Fabbri & Callin, 2010).

In summary, epigenetics is one of the most promising fields in biomedical research. Novel strategies for risk assessment, early detection and new therapeutic targets may be revealed by epigenetic studies (Boumber & Issa, 2011). This chapter will summarize the common epigenetic aberrations that are detected in bladder cancer, their translational implications and possible epigenetic therapies.

2. Translational implications of epigenetic changes in bladder cancer

Bladder cancer is the fifth most commonly diagnosed non-cutaneous solid tumor and the second most common in the urological tract. Although many tumors that originate in this organ are superficial, with low risks of metastasis, bladder cancer has a high recurrence risk; the 4-year recurrence rate for patients with superficial tumors is 50%. Currently, the diagnosis of bladder cancer is based on histological, pathological and morphological parameters and provides only a generalized outcome for patients (Tanaka & Sonpadvde, 2011). The gold standard for detecting bladder cancer is cystoscopic examination, but this analysis is costly, causes discomfort to the patient (invasive method) and has variable sensitivity, providing only a generalized outcome to patients. In addition, the sensitivity of the cytological analysis is questionable, especially in cases of low-grade carcinoma (Kim & Kim, 2009). With the advent of targeted therapy, molecular biomarkers are becoming increasingly important in both clinical research and practice. These markers are being identified with the purpose of reducing the need for invasive follow-up examinations and also to anticipate the prognosis of individual patients. Furthermore, the early diagnosis of

bladder cancer by non-invasive methods could allow for more effective treatment and optimize the success of surgical therapy.

The DNA methylation of CpG islands that are mapped to promoter regions of specific genes, such as tumor suppressor genes, has been extensively reported in many cancer types. In bladder cancer, this epigenetic event has been related to tumor development, staging, recurrence, progression and clinical outcome. More specifically, DNA methylation has been strongly associated with higher stages, high rates of tumor progression and high mortality in patients with this cancer. As was demonstrated by Wolff et al. (2010), the analysis of epigenetic backgrounds can allow for the differentiation between noninvasive and invasive tumors by the identification of the different epigenetic characteristics that are present, such as the extensive DNA hypomethylation that is observed in noninvasive tumors compared to the high rates of DNA hypermethylation in invasive urothelial cancer. This may explain why ~15% of tumors will progress to invasive disease and have poor prognosis, while others will remain with low rates of generate metastasis.

Currently, some histone modifications and the aberrant expression of miRNAs have been linked to tumorigenesis and have also been identified to be reliable and strong biomarkers for bladder cancer. MicroRNAs are specifically interesting because they are very stable in body fluids due to their small sizes and thus are resistant to degradation by nucleases, which are present in large quantities in urine (Tilki et al., 2011).

2.1 Candidate epigenetic biomarkers in the diagnosis of bladder cancer

Because DNA methylation is chemically and biologically stable and can be detected early in the carcinogenesis process, this epigenetic change has been considered to be a valuable potential diagnostic marker that is feasible to assess in clinical routine analysis through the investigation of exfoliated cells in the urine or blood of patients with bladder cancer and appears to be more sensitive than conventional cytology. A number of genes have been identified as being hypermethylated in the urine or tissue samples of cancer patients compared to healthy tissues, indicating that the down-regulation of these genes has some clinical relevance to the origin and development of the disease (Table 1).

One example is the *RUNX3* (runt-related transcription factor 3) gene, which has been mapped to 1p36 and is thought to be a tumor suppressor gene that is frequently deleted or transcriptionally silenced in patients with cancer. In a study that analyzed 124 tumor tissue samples, 73% were found to have a methylation-positive pattern compared to the methylation-free pattern that was exhibited by the normal bladder mucosa. Moreover, the methylation of this gene was found to confer a significant increase (100-fold) in the risk of tumor development (Kim et al., 2005), suggesting that it may have potential as a potent bladder cancer detection marker.

Our group also contributed to the literature surrounding epigenetic markers in bladder cancer. We discovered high rates of DNA methylation in exfoliated urinary cells, in which the *RARB* gene had a sensitivity of 95% and specificity of 71% for detecting the presence of cancer (Negraes et al., 2008). These results are concordant with the increased methylation frequencies that have been previously described (Chan et al., 2002; Hoque et al., 2006) and suggest that this gene could be considered as a diagnostic biomarker. It encodes a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators that binds retinoic acid (the biologically active form of vitamin A) and also mediates cellular signaling during embryonic morphogenesis and cell growth and differentiation. It is

thought that this protein limits the growth of many cell types by regulating gene expression (Soprano et al., 2004).

In the study conducted by Renard et al. (2010), it was demonstrated that 2 genes (*TWIST1* and *NID2*) were frequently methylated in urine samples collected from bladder cancer patients, including those with early-stage and low-grade diseases, with a specificity of 93% and sensitivity of 90%, which was an improvement from the cytological method of detection (48%).

Besides the identification of DNA hypermethylation at a single *locus*, some authors have demonstrated that several genes may be analyzed together to generate a profile of hypermethylated genes. These profiles may be able to allow for a more sensitive and reliable marker for the detection of bladder cancer (Table 1). Based on this, Chan et al. (2002) discovered that the sensitivity of the methylation analysis (90.9%) of four genes (*DAPK1, RARB, CDH1* and *CDKN2A*) was higher than that of urine cytology (45.5%) for cancer detection and was more striking in low-grade cases (100% versus 11.1%).

Similarly, Urakami et al. (2006) found that the identification of the increased methylation of six Wnt-antagonist genes (*SFRP1, SFRP2, SFRP4, SFRP5, WIF1* and *DKK3*) could predict bladder tumors with a sensitivity of 77.2% and specificity of 66.7%. These genes are known to inhibit Wnt signaling by binding to specific molecules that act in this pathway. The DNA methylation and consequent functional loss of these genes may result in the activation of the Wnt signaling pathway and promote the dysregulation of cell proliferation and differentiation. The authors also discovered that two of these genes (*SFRP2* and *DKK3*) were able to act as independent predictors of bladder tumors ($P < 0.05$ and $P < 0.01$, respectively).

Friedrich et al. (2004) also suggested that the presence of a combination of DNA methylation at the 5′ regions of three apoptosis-associated genes (*DAPK1, BCL2* and *TERT*) in urine sediment could be diagnostic of bladder cancer with a sensitivity of 78%, suggesting that this combined methylation analysis was a highly sensitive method for the noninvasive detection of bladder cancer.

In addition, Hoque et al. (2006) proposed a two-stage predictor for the classification of bladder cancer that was based on an investigation of a panel composed of nine genes (*APC, ARF, CDH1, GSTP1, MGMT, CDKN2A, RARB, RASSF1A* and *TIMP3*) in urine sediment. In the first stage, patients who presented with DNA methylation in the promoters of at least one of four specific genes (*CDKN2A, ARF, MGMT* and *GSTP1*) were classified as having cancer (100% specificity). Moreover, patients with no methylation in these genes were subjected to a second stage of investigation with a logistic prediction of risk scores based on the promoter methylation of the five remaining genes (sensitivity of 82% and specificity of 96%).

Three of these genes had previously been investigated by Dulaimi et al. (2004), who demonstrated the feasibility of obtaining reproducible highly sensitive (87%) and 100% positive identifications of hypermethylation in a panel composed of the *APC, RASSF1A* and *CDKN2A* tumor suppressor genes in urine in cases of early-stage disease. In addition to *RASSF1A* (a tumor suppressor gene that is frequently inactivated in several cancer types), the other two genes chosen were involved in the $p53/p14^{ARF}$ tumor suppressor gene pathway (*CDKN2A* gene) (Sherr & McCormick, 2002) and the Wnt signaling pathway (*APC* gene) (Taipale & Beachy, 2001). The evaluation of this panel yielded superior results compared to those of cytology in the detection of bladder cancer. Yates et al. (2006) also investigated the *APC, RASSF1A* and *CDH1* genes in urine. This panel generated a lower sensitivity (69%) and specificity (60%) than the former; however, the diagnostic accuracy was 86%.

Many of the genes that have been chosen to be investigated in combined analyses to generate panels have frequently been suggested to be individually methylated in bladder cancer, such as *RASSF1A*. The DNA methylation of this gene had previously been reported to be able to detect bladder cancer in urine samples with 100% sensitivity by Chan et al. (2003). The authors advocated that the detection of gene methylation using multiple markers could increase both the sensitivity and specificity of cancer detection, and the addition of *RASSF1A* to this panel could improve the diagnostic accuracy even further.

Yu et al. (2007) discovered that the methylation of a panel composed by 11 genes (*SALL3, CFTR, ABCC6, HPSE, RASSF1A, MT1A, ALX4, CDH13, RPRM, APBA1* and *BRCA1*) in urine sediments showed positive correlations with diagnosis in 121 out of 132 bladder cancer cases with a sensitivity of 91.7% and accuracy of 87%. Remarkably, this approach was able to detect more than 75% of tumors at stage 0a and 88% of stage I tumors, indicating the value of this panel in the early diagnosis of bladder cancer.

Likewise, a three-gene (*GDF15, TMEFF2* and *VIM*) panel was able to detect bladder cancer in urine samples with a sensitivity of 94% and specificity of 100% (Costa et al., 2010), exceeding the detection rates that are normally obtained using conventional cytopathology and cytology. This panel of genes was selected based on stringent criteria after a screening test that employed a genome-wide approach and was distinctive because it was able to detect bladder cancer by noninvasive methods even when patients with kidney or prostate cancer were used as controls. These three genes are biologically relevant to carcinogenesis because *TMEFF2* (mapped at 2q32.3) and *VIM* (mapped at 10p13) were previously found to be silenced by promoter methylation in esophageal, colorectal (Shirahata et al., 2009; Tsunoda et al., 2009) and bladder cancer (Hellwinkel et al., 2008). *GDF15* (mapped to 19p13.11) is a member of the transforming growth factor (TGF)-β superfamily and may act as a tumor suppressor gene in early-stage cancers (Eling et al., 2006).

Another biomarker of interest for the detection of urothelial cancer, according to Ellinger et al. (2008), is cell-free serum DNA methylation. The authors detected that the diagnostic accuracy of this marker increased when hypermethylation at multiple gene sites was assessed simultaneously, particularly at the *GSTP1, RARRES1* or *APC* genes (80% sensitivity and 93% specificity).

The list of aberrant epigenetically regulated genes continues to grow. Is important to note that the same genes have been investigated by different groups, and the methylation rates found may vary from one report to another. Results may reflect the distinct methodologies employed, the numbers and types of samples (urine, surgical tissue and/or serum) as well as disease classifications. Nevertheless, the reports above highlight the high potential of DNA methylation markers for the effective early detection of bladder cancer using noninvasive urine tests.

The measurement of global cytosine methylation rates (%5-mC) concomitantly with the DNA methylation of specific genes could be a useful biomarker to assess a patient's susceptibility to bladder cancer. In a large case-control study conducted by Moore et al. (2008), the DNA hypomethylation of leukocytes was strongly associated with an increased bladder cancer risk, and this association was independent of smoking and other assessed risk factors.

Recently, evidence has emerged that circulating miRNAs are present in human body fluids (as urine) in concentrations that are subject to variation during cancer pathogenesis or development (Iguchi et al., 2010), as was reported by Dudziec et al. (2011). The authors discovered that the combined low expression levels of miR-152, -328 and -1224-3p allowed

for accurate diagnosis with 81% sensitivity and 75% specificity. These miRNAs were found to be epigenetically regulated by DNA methylation at CpG islands and the shores (regions of less dense CpG dinucleotides) that surrounded them following a genome-wide screening.
In addition, Hanke et al. (2010) found that the ratio of miR-126:miR-152 enabled the detection of bladder cancer from urine samples with a specificity of 82% and a sensitivity of 72%; thus, they may be used as a tumor markers for this disease.
In addition, Yamada et al. (2011) identified one microRNA (miR-96) that may be a useful diagnostic marker with high sensitivity and specificity (71.0% and 89.2%, respectively) when assessed in combination with urinary cytology (80% diagnostic accuracy). This molecule, which has been mapped to 7q32, is a putative onco-miRNA that has been demonstrated to be able to down-regulate tumor suppressor genes. It was found to be up-regulated in a previous study conducted by the same group (Ichimi et al., 2009), in which the microRNA expression signatures that are specific to bladder cancer were determined, and a subset of 7 microRNAs (miR-145, miR-30a-3p, miR-133a, miR-133b, miR-195, miR-125b and miR-199a*) that are significantly down-regulated in bladder cancer were validated. These microRNAs were sufficiently sensitive (>70%) and specific (>75%) to distinguish bladder cancer from normal epithelium.
As mentioned above, not only the DNA methylation but also the expression profile of the miRNA molecules have been closely associated with the diagnosis of bladder cancer.

Epigenetic biomarker	Samples	Sensitivity/ specificity/ OR	Supporting literature
DNA methylation			
RASSF1A	Urine	Sensitivity: 100%	Chan et al., 2003
RUNX3	Tissue	OR 107.55 (95% CI, 6.33-1827.39)	Kim et al., 2005
RARB	Bladder washing (exfoliated cells)	OR/Sensitivity/specificity: 48.89/95%/71%	Negraes et al., 2008
TWIST1 and *NID2*	Urine	Sensitivity/specificity: 90%/93%	Renard et a., 2010
DAPK1, RARB, CDH1 and *CDKN2A*	Urine	Sensitivity/specificity: 90.9%/76.4%	Chan et al., 2002
APC, RASSF1A and *CDKN2A*	Urine	Sensitivity/specificity: 87%/100%	Dulaimi et al., 2004
DAPK1, BCL2 and *TERT*	Urine	Sensitivity: 78%	Friedrich et al., 2004
APC, ARF, CDH1, GSTP1, MGMT, CDKN2A, RARB, RASSF1A and *TIMP3*	Urine	1st stage sensitivity: 100% 2nd stage Sensitivity/specificity: 82%/96%	Hoque et al., 2006
SFRP1, SFRP2, SFRP4, SFRP5, WIF1, DKK3	Tissue	Sensitivity/specificity: 77.2%/66.7%	Urakami et al., 2006
APC, RASSF1A and	Urine	Sensitivity/specificity:	Yates et al., 2006

Epigenetic biomarker	Samples	Sensitivity/ specificity/ OR	Supporting literature
CDH1		69%/60%	
SALL3, CFTR, ABCC6, HPSE, RASSF1A, MT1A, ALX4, CDH13, RPRM, APBA1 and *BRCA1*	Urine	Sensitivity/especificity: 91.7%/87%	Yu et al., 2007
GSTP1, RARRES1, APC	Cell-free serum DNA	Sensitivity/specificity: 80%/93%	Ellinger et al., 2008
GDF15, TMEFF2 and *VIM*	Urine	Sensitivity/specificity: 94%/100%	Costa et al., 2010
%5-mC of leukocytes	Blood cells	OR 1.38 (95% CI:1.05–1.08, p=0.02)	Moore et al., 2008
miRNAs			
miR-145, miR-30a-3p, miR-133a, miR-133b, miR-195, miR-125b and miR-199a*	Tissue	Sensitivity/specificity: >70%/>75%	Ichimi et al., 2009
RNA ratio of miR-126:miR-152	Urine	Sensitivity/specificity: 72%/82%	Hanke et al., 2010
miR-152, -328 and -1224	Urine	Sensitivity/specificity: 81%/75%	Dudziec et al., 2011
miR-96	Urine	Sensitivity/specificity: 71%/89.2%	Yamada et al., 2011

Table 1. Epigenetic diagnostic markers in bladder cancer. The genes were described as official symbols according recommendations of Guidelines for Human Gene Nomenclature. More information about specific genes can be achieved at http://www.genenames.org/guidelines.html.

2.2 Candidate epigenetic biomarkers in the prognosis of bladder cancer

The knowledge of prognostic factors is of great importance for the determination of therapeutic strategies and to enable the application of different modalities of therapy in cancer treatment. In cases of bladder cancer, patients are monitored for recurrence or progression by periodic cystoscopy and urine cytological analysis, the frequencies of which vary depending on the risk factors that are associated with the disease. Thus, the discovery of more sensitive and non-invasive tumor markers that can help to predict tumor recurrence, progression and metastasis are required, and epigenetic alterations may be promising new potential prognostic markers for bladder cancer.

Bladder tumors may be superficial, with low risks of metastasis, but may have high recurrence risks (McConkey et al., 2010). Several genes that are related to the progression and prognosis of bladder cancer have been identified in bladder washes, urine and tumor tissues using various molecular and epigenetic approaches (Mitra et al., 2006) and are considered to be potential markers (Table 2).

Maruyama et al. (2001) determined the methylation statuses of 10 genes in 98 fresh bladder tumor tissues and found that multiple genes are methylated during the process of bladder

cancer development. Their results also indicated that the frequent methylation of four genes (*CDH1*, *CDH13*, *RASSF1A* and *APC*) together with high MIs (median methylation index) were correlated with poor prognosis (tumors showed high grade, nonpapillary growth patterns, muscle invasions, advanced tumor stages and aneuploidies). In addition, the methylation of *CDH1*, *FHIT* and high MIs were associated with reduced patient survival rates.

In a study performed by Catto et al. (2005) that employed a large cohort of urothelial carcinomas, CpG hypermethylation at *DAPK* was associated with higher progression rates (log-rank P = .014) in all of the transitional-cell carcinoma (TCC) samples that were investigated compared to unmethylated samples at this *locus*.

In another study, Christoph et al. (2006) selected related genes as targets of p53 in the apoptotic cycle to perform a quantitative analysis of 110 tumor samples. The authors found that *APAF1* methylation levels were correlated with tumor stages and grades. In addition, the methylation levels of the *APAF1* and *IGFBP3* genes enabled tumors with higher recurrence risks to be distinguished from low-risk tumors in non-muscle-invasive and muscle-invasive tumors. The epigenetic inactivation of pro-apoptotic genes may be important events that are related to the progression and increased aggressiveness of tumors that are hypermethylated in these *loci*.

In addition, the hypermethylation of the promoter region of the *TIMP3* gene detected in urine sediments was found to be associated with an increased risk of death (Hoque et al., 2008). Other genes also have been found to undergo aberrant promoter methylation and were associated with poor prognosis in bladder cancer, including the hypermethylation of the *RUNX3* promoter, which was correlated with the development of invasive tumors, tumor progression and cancer specific-survival in patients with TCC (Kim et al., 2008). The methylation of this gene was also shown to be related to an increased risk of developing bladder cancer (Kim et al., 2005), suggesting that this gene not only suppresses the aggressiveness of tumors but also inhibits the tumor development.

Beyond to the tumor size and grade parameters, response to treatment is also an important prognostic factor because multidrug resistance to chemotherapy is a major obstacle in the treatment of cancer patients. Tada et al. (2000) showed that the overexpression of the *ABCB1* gene may be a prognostic factor indicating recurrence in bladder cancer, and the hypomethylation of the promoter of this gene may be necessary for the development of increased *ABCB1* mRNA levels and multidrug resistance.

Global DNA hypomethylation is also a common phenomenon that has been reported in bladder cancer (Seifert et al., 2007). The loss of DNA methylation in repetitive sequences may account for a majority of the global hypomethylation that characterizes a large percentage of human cancers. Neuhausen et al. (2006) found that the hypomethylation of LINE-1 retrotransposons was present in 90% of the urothelial carcinoma specimens that were studied, and the absence of this epigenetic change was indicative of a better clinical prognosis. In a high-throughput DNA methylation analysis, a distinct hypomethylation pattern was found in non-invasive (Ta-T1) urothelial tumors compared to both normal urothelium and invasive tumors (Wolff et al., 2010). These researchers found a substantial number of probes to be hypomethylated in non-invasive tumors only, suggesting that lower levels of DNA methylation may be related to a less malignant phenotype.

A particularly interesting example of epigenetic regulation is genomic imprinting, in which one copy of a gene is silenced in a manner determined by its parental origin. Thus, imprinted genes show parental-specific monoallelic expression. The loss of allele-specific

expression pattern is termed as loss of imprinting (LOI), an event described in several types of pediatric and adult cancers (Monk, 2010). LOI has already been identified as an

Clinical – histolopathological parameters	Epigenetic biomarker	Supporting literature
Grade	**DNA methylation** *CDKN2A, BCL2, TERT, EDNRB, CDH1, RASSF1A, APC, CDH13*	Maruyama et al., 2001; Domínguez et al., 2002; Friedrich et al., 2004
Stage	**DNA methylation** *TIMP3, CDKN2A, RASSF1A, BCL2, OPCML, CDH1, APC, CDH13* **Histone modification** H4K20me1	Maruyama et al., 2001; Domínguez et al., 2002; Friedrich et al., 2004; Hoque et al., 2008; Schneider et al. 2011; Duarte-Pereira et al., 2011
Recurrence	**DNA methylation** *DAPK1, H19, TIMP3*	Ariel *et al*., 2000; Tada et al., 2002; Friedrich et al., 2005
Survival	**DNA methylation** *TIMP3, OPCML, RUNX3, FHIT, CDH1* **Histone modification** H4K20me3	Maruyama et al., 2001; Kim et al., 2008; Hoque et al., 2008; Schneider et al. 2011; Duarte-Pereira et al., 2011
Metastasis	**DNA methylation** *TIMP3* **miRNA expression** miR-452, miR-452*	Hoque et al., 2008; Veerla et al., 2009
Muscle invasion	**DNA methylation** *CDKN2A, CDH1, RASSF1A, APC, CDH13* **miRNA expression** miR-222, miR-125b	Maruyama et al., 2001; Domínguez et al., 2002; Veerla et al., 2009
Tumor progression	**DNA methylation** *RASSF1A, CDH1, TNFRSF25, EDNRB, APC, DAPK1, H19* **Histone modification** H3K4me1, H4K20me1, H4K20me2, H4K20me3 **miRNA expression** Set of miR-21, miR-510, miR-492, miR-20a, miR-198 and set of miR-455-5p, miR-143, miR-145, miR-125b, miR-503	Catto et al., 2005; Yates et al., 2007; Dyrskjøt et al., 2009; Schneider et al. 2011

Table 2. Epigenetic prognostic markers in bladder cancer. The genes were described as official symbols according recommendations of Guidelines for Human Gene Nomenclature. More information about specific genes can be achieved at http://www.genenames.org/guidelines.html.

epimarker of cancer development. The *IGF2* and *H19* imprinted genes have been well documented in the literature. Some studies showed that the *H19* gene is involved in the development of bladder cancer (Ariel et al., 1995; Elkin et al., 1995) and is associated with high recurrence risks for this tumor type (Ariel et al., 2000). Furthermore, insulin-like growth factor-II (IGF-II) loss of imprinting (LOI) in a series of paired tumoral and normal adjacent bladder tissues and E-cadherin (*CDH1*) immunolocalization suggested a possible mechanism underlying E-cadherin relocalization to the cytoplasm, that is, the presence of aberrant levels of IGF-II due in some cases to *IGF2* LOI (Gallagher et al., 2008). Furthermore, the finding of LOI in the tumoral adjacent normal samples holds promise of *IGF2* LOI as a predictor of tumor development.

Others epigenetic mechanisms in cancer patients remain less comprehensively understood. One of these epigenetic changes involves the histone modifications, which include changes in their levels and distribution at gene promoters, gene coding regions, repetitive DNA sequences and other genomic elements (Kurdistani, 2011). In a recent study, Schneider et al. (2011) found that global levels of H3K4me1, H4K20me1, H4K20me2 and H4K20me3 were decreased compared to normal urothelium. The distribution of these histone modifications were associated with the risk of metastasis in muscle-invasive compared to non-muscle-invasive bladder cancers. The authors also showed that H4K20me1 levels were increased in patients with non-muscle-invasive bladder cancer with advanced pT stages and less differentiated bladder cancer, and H4K20me3 levels were significantly correlated with mortality after radical cystectomy in patients with muscle-invasive cancer.

Recently, several groups have questioned whether the miRNA expression profiles or even single miRNAs could act as useful biomarkers not only for cancer diagnosis but also for prognosis and treatment optimization (Lu et al., 2005; Calin & Croce, 2006). Dyrskjøt et al. (2009) identified the aberrant expression of several miRNAs in 106 samples from patients with different stages of bladder cancer and associated their profiles with disease progression. Among the miRNAs that were differentially expressed in normal bladder tissue compared to that of bladder cancer, two subsets [(miR-21, miR-510, miR-492, miR-20a, miR-198) and (miR-455-5p, miR-143, miR-145, miR-125b, miR-503)] were up- and down-regulated by two-fold, respectively. In another large-scale study that evaluated miRNA expression, high expression levels of miR-222 and miR-125b were observed in muscle-invasive tumors, and miR-452 and miR-452* were shown to be over-expressed in node-positive tumors (Veerla et al., 2009).

Moreover, aberrant DNA methylation has been implicated in the deregulation of several miRNAs in different types of cancer (Lujambio et al., 2007). Wiklund et al. (2011) studied this relationship and found that the miR-200 family and miR-205 are concurrently silenced and that DNA hypermethylation would be associated with the silencing of these microRNAs in invasive bladder tumors. They also found that the loss of miR-200c expression was associated with disease progression of muscle-invasive cancers and with poor prognosis.

3. The promise of epigenetic therapy

The knowledge of epigenetic alterations that are associated with human cancers and their potential reversibility has prompted the development of drugs that target epigenetic enzymes. Either natural or synthetic modulators can be utilized to restore normal epigenetic and gene expression patterns; for example, by restoring the expression of the frequently

silenced *RUNX3* gene, which is considered to be good target for this new therapeutic modality since the loss of its function in cancer cells due to genetic mutations is a rare event (Kim et al., 2005). The epigenetic therapy can be used alone or in combination with other therapeutic modalities, such as chemotherapy, immunotherapy or radiotherapy. This approach will eventually lead to targeted therapies that are suited for specific molecular defects, thereby significantly decreasing the morbidity associated with bladder cancer in addition to other cancers (Balmain, 2002; Kim & Kim, 2009; Mund & Lyko, 2010).

Two principal classes of epigenetic drugs have been demonstrated to be clinically relevant: DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors (Esteller, 2005) (Table 3). Novel epigenetic compounds that are of potential interest as clinical therapeutic drugs include the histone acetyltransferase inhibitors, such as anacardic acid, curcumin and peptide CoA conjugates. In addition, histone methyltransferase inhibitors and HDACis that are specific for SIRT1 (class III HDAC), such as nicotinamide and splitomycin, are now under intense analysis (Ballestar & Esteller, 2008; Greiner et al., 2005).

3.1 DNMTs inhibitors

Genes that are silenced by DNA hypermethylation may be reactivated by small molecules that are called DNMT inhibitors. These agents may be structural analogues of the nucleoside deoxycytidine or non-nucleoside analogues. The analogues, after being phosphorylated by kinases that convert the nucleosides into nucleotides, can be incorporated into DNA and subsequently inhibit DNMT activity by forming a covalent bond with the cysteine residue in the active DNMT site. However, it has also been shown that such incorporation may lead to instabilities in DNA structure and even DNA damage (Bouchard & Momparler, 1983; Goffin & Eisenhauer, 2002).

Two prominent examples are the cytosine analogs 5-azacytidine (azacytidine, Vidaza) and 2'-deoxy-5-azacytidine (decitabine, Dacogen), which are potent inhibitors of DNMTs (Table 3) and have been approved by the FDA (Food and Drug Administration) for the treatment of myelodysplastic syndrome, a pre-leukemic bone marrow disorder (Lübbert, 2000). Various additional molecules has been found to possess better stability and less toxicity and are currently being investigated as DNMT inhibitors in preclinical experiments, such as dihydro-5-azacytidine, arabinofuranosyl-5-azacytosine (fazarabine) and zebularine (Cheng et al., 2003).

Azacytidine and decitabine have been widely used in cell culture systems to reverse DNA hypermethylation and restore silenced gene expression. However, results from *in vivo* studies are not satisfactory, especially with solid tumors in which limited efficacy has been encountered. In general, both agents are unstable in aqueous solutions, have short half-lives and need to be freshly prepared before administration. In addition, both drugs have relatively poor bioavailabilities and high cytotoxic effects with potential risks, such as myelotoxicity, mutagenesis, and tumorigenesis, which have limited their clinical applications (Jackson-Grusby et al., 2007).

Despite this discouraging data, the orally administered zebularine shows some promise. It was shown to suppress the growth of TCC in bladder xenografts in nude mice and was less toxic than other nucleoside analogues. In addition, when zebularine was given at a lower dose after an initial dose of decitabine, a profound demethylation of the *CDKN2A* gene promoter was observed. These results provide a rationale for the strategy of combining an

initial administration of a parenteral DNMT inhibitor with a subsequent low dose of oral zebularine (Cheng et al., 2004; Zhang et al., 2006).

Another group of compounds are called non-nucleoside analogues. These small molecules inhibit DNA methylation by binding directly to the catalytic site of the DNMT enzyme without being incorporated into the DNA. The local anesthetic procaine and its derivative procainamide, which is an approved antiarrhythmic drug, have exhibited demethylating activities. For example, Lin et al. (2001) reported that procainamide was able to restore *GSTP1* gene expression by reversing the hypermethylation of the promoter CpG islands of androgen-sensitive human prostate adenocarcinoma (LNCaP) cells *in vitro* and *in vivo*. Because these agents do not incorporate into DNA, it is expected that they may have less genotoxicity than nucleoside DNMT inhibitors. In addition, (-)-epigallocatechin-3-gallate (EGCG), the main polyphenol compound in green tea, also acts as DNMT inhibitor . Cancer cells treated with micromolar concentrations of EGCG showed reduced DNA methylation and the increased transcription of tumor suppressor genes. However, it is still unknown whether EGCG has a direct inhibitory effect on DNMTs (Fang et al., 2003; Villar-Garea et al., 2003).

3.2 HDAC inhibitors

A variety of structurally distinct groups of compounds have been identified as histone deacetylase inhibitors (HDACi) (Table 3). These compounds inhibit histone deacetylase activity by binding to the catalytic site of the enzyme and chelating zinc ions because they share similar structures with the substrates (Finnin et al., 1999). Similar to their effects on gene expression and differentiation, HDACi have also been shown to be efficient inducers of apoptosis in several cellular systems. The precise mechanism of this effect is under investigation, and it has been suggested that they may affect cellular oxidative stress and DNA damage induction. They have shown impressive activities in preclinical studies as well as selectivity for neoplastic cells. Many HDACi are being tested in clinical trials for various malignancies (Bolden et al., 2006; Xu et al., 2007).

The class of the HDAC inhibitors is divided into four groups: hydroxamic acids, cyclic tetrapeptides, short-chain fatty acids and benzamides. The hydroxamate compounds are more potent and have higher inhibitory effects. Trichostatin A from *Streptomyces hygroscopicus* is active at nanomolar concentrations, while the synthetic compounds, such as suberoylanilide hydroxamic acid (SAHA), can function in low micromolar or nanomolar ranges. Cyclic tetrapeptides are very potent compounds and can inhibit histone deacetylase at nanomolar concentrations. Short-chain fatty acid compounds usually require millimolar concentrations to inhibit histone deacetylase activities *in vivo*; therefore, their clinical applicability could be limited. The fourth class is the benzamides, such as MS-275 and CI-994, which are effective at micromolar concentrations (Rosato et al., 2003; Zhang et al., 2006).

The clinical potentials of histone deacetylase inhibitors have been suggested by several promising *in vivo* studies. For example, SAHA was FDA approved in Oct. 2006 for the treatment of cutaneous T cell lymphoma (CTCL), and it is under a phase I clinical trial for use in patients with TCC (Mann et al., 2007). Preliminary reports have indicated that 2 out of 6 patients with metastatic TCC disease have had objective tumor regression and tumor-related symptom relief (Kelly et al., 2003). The induction of *CDKN1A* messenger RNA and

Group	Drug	Clinical status
DNMT inhibitor		
Nucleoside analogues	5-Azacyticine	Approved 2004 for MDS
	2´-Deoxy-5-azacytidine	Approved 2006 for MDS
	Zebularine	Preclinical
Non-nucleoside analogues	Hydralazine	Phase I > II for cervical Ca
	MG98	Phase I > II for advanced metastatic tumors
	Procaine	Preclinical
	RNAi	Preclinical
	Epigallocatichin-3-gallate	Preclinical
	Psammaplin A	Preclinical
HDAC inhibitor		
Hydroxamic acids	Suberoylanilide hydroxamic acid (SAHA)	Approved 2006 for CTCL
	Panobinostat	Phase I > II > III for breast Ca, gliomas, prostate Ca, NSCLC, CTCL, leukemia
	Belinostat	Phase I > II for ovarian Ca, CTCL, lymphoma, multiple myeloma, leukemia
	Trichostatin A	Preclinical
Cyclic tetrapeptides	Depsipeptide, Romidepsin	Approved 2009 for CTCL
Short-chain fatty acids	Valproic acid	Phase I > II > III for melanoma, myelodysplastic syndrome, leukemia, chronic lymphocytic leukemia, cervical Ca, breast Ca
	AN-9	Phase I > II for malignant melanoma, leukemia, lymphoma, NSCLC
Benzamides	Entinostat	Phase I > II for breast Ca, acute lymphoblastic leukemia, Hodgkin's lymphoma, MDS, renal Ca, colorectal Ca, lung Ca
	Mocetinostat	Phase I > II for breast Ca, NSCLC, prostate Ca, stomach Ca, non-Hodgkin's lymphoma, Hodgkin's lymphoma, AML, CLL, lymphoma
	N-Acetyldinaline	Phase I > II > III for multiple myeloma, lung Ca, pancreatic Ca

Table 3. List of the main DNMT and HDAC inhibitors and their current clinical trial status**.** MDS: myelodysplastic syndrome, CTCL: cutaneous T cell lymphoma, NSCLC: non small cell lung cancer, AML: acute myeloid leukemia, CLL: Chronic lymphocytic leukemia, Ca: cancer. Clinical status source: clinicaltrials.gov.

protein levels in T24 cells following SAHA exposure mediated by increased acetyl H3 and H4 levels in the respective promoter region may contribute to its tumor inhibitory effect

(Richon et al., 2000). Other researchers have reported similar inhibitory effects on bladder tumor growth using trichostatin A and pyroxamide on T24 cells. Additionally, trichostatin A is able to suppress 70% of tumor growth with no detectable toxicity in EJ and UM-UC-3 xenograft models (Canes et al., 2005).

3.3 Combination therapy

The emerging concept of gene silencing involves the interaction of multiple factors that may act in a sequential manner. It is also known that a single agent may not be able to eradicate a tumor mass that is derived from a very heterogeneous population of cells. Moreover, the adverse toxic effects that are caused by single-agent treatments, especially at high doses, call for a rationalized therapeutic approach with low-dosage drug combinations. Accumulating evidence has shown that the combination of histone deacetylase inhibitors and DNMT inhibitors is very effective (and synergistic) in inducing apoptosis, differentiation and/or cell growth arrest in various human cancer cell lines (Gottlicher et al., 2001; Mei et al., 2004; Stirzaker et al., 2004).

In urologic cancers, Cameron et al. (1999) showed that the combination of decitabine and trichostatin A stimulated a synergistic reactivation of several tumor suppressor genes. Dunn et al. (2005) reported that the combination of DNMT inhibitors and histone deacetylase inhibitors was able to reactivate the sensitivities of LNCaP cells to interferon treatment by the re-expression of JAK1 kinase, which is a key mediator of both interferon-gamma and interferon-alpha/beta receptor-elicited effects. Another strategy is to combine either histone deacetylase inhibitors or DNMT inhibitors with conventional therapies, as was demonstrated by Zhang et al. (2007), who indicated that the combination of FK228 (a HDAC inhibitor) and docetaxel (chemotherapeutic drug) caused a synergistic growth inhibition in androgen-independent prostate cancer cell lines. Moreover, single treatments with SAHA or MS-275 show enhanced radiation-induced cytotoxicity in DU-145 cells both *in vitro* and *in vivo* (Chinnaiyan et al., 2005).

4. Future

There is a great deal of evidence that demonstrates the connections between epigenetic modification enzymes and cancer. Epigenetic alterations contribute to tumorigenesis by the activation of oncogenes or inactivation of tumor suppressor genes. The identification of molecules that can modulate epigenetic enzymes could lead to the prevention of oncogene transcription and activation of tumor repressors, and thus it is an important topic to research (Zheng, 2008).

A major impediment to the use of such drugs is that they are nonspecific and may reactivate genes non-discriminately. However, this does not seem to be a problem in the present case because DNA methylation inhibitors only act on dividing cells and leave normal, non-dividing cells unaffected. Also, it seems that the drugs preferentially activate genes that have become abnormally silenced in cancer. Further studies are required to establish an unambiguous proof of concept for epigenetic cancer therapies (Jones & Baylin, 2007; Liang et al., 2002; Mund & Lyko, 2010).

For future clinical applications, researchers should focus on several aspects, including the biomarkers that predict drug responses. Researchers should also focus on the screening of

new, more effective and less toxic agents. The psammaplin, for example, a family of bromotyrosine derivatives that have been extracted from the marine sponge *Pseudoceratina purpurea,* appear to be a novel class of compounds with the ability to inhibit both DNMT and histone deacetylase activities (Pina et al., 2003).

In addition, exploring the silencing of specific genes by RNA interference for key epigenetic regulatory complexes could enhance therapeutic indices. For example, DNMT-specific siRNA (single-interfering RNA) is able to elicit the demethylation of several epigenetically silenced genes. Additionally, the treatment of cultured cells *in vivo* with demethylating agents, either alone or in combination with HDACi, has been shown to re-activate the expression of tumor-suppressor miRNAs, such as miR-124a and miR-127, causing the corresponding repression of their oncogenic targets. Although the successful delivery of siRNAs to solid tumors has yet to be achieved, designing small-molecule siRNAs to mimic tumor-suppressor miRNAs could be a potential method to selectively repress the expression of oncogenes (Leu et al., 2003; Saito et al., 2006).

In the next decade, with the availability of gene profiling databases of epigenetic modifiers, it is expected that epigenetic therapy will be translated from the bench to the clinical arena and become a real alternative to conventional cancer treatments (Rodríguez-Paredes & Esteller, 2011; Zhang et al., 2006).

In summary, the field of epigenetic biomarker studies is still new but shows promise in the clinical management of cancer. Valuable progress has been made on this end, and the combination of existing and newly discovered biomarkers will likely allow for more accurate diagnosis. Thus, patients will be able to benefit from this new era of personalized medicine, in which biomarkers will allow for direct treatments with more effective therapeutic agents.

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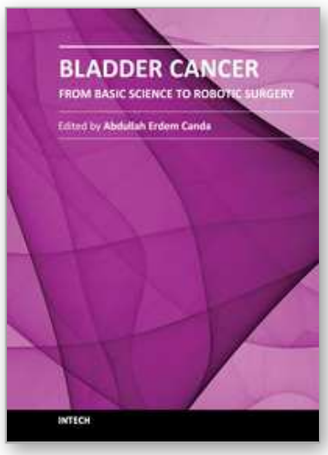
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This book is an invaluable source of knowledge on bladder cancer biology, epidemiology, biomarkers, prognostic factors, and clinical presentation and diagnosis. It is also rich with plenty of up-to-date information, in a well-organized and easy to use format, focusing on the treatment of bladder cancer including surgery, chemotherapy, radiation therapy, immunotherapy, and vaccine therapy. These chapters, written by the experts in their fields, include many interesting, demonstrative and colorful pictures, figures, illustrations and tables. Due to its practicality, this book is recommended reading to anyone interested in bladder cancer.

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