Title: *The Prognostic Role of Epigenetic dysregulation in Bladder Cancer: A Systematic Review*

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HIGHLIGHTS

- Epigenetic dysregulation is of potential prognostic value in bladder cancer.
- Prognostic epigenetic alterations involve known cancer-related cellular processes.
- A predominantly repressive chromatin state might be an adverse prognostic factor.
- Epigenetic biomarkers need to be prospectively evaluated in bladder cancer.

ABSTRACT

Background: Despite adequate treatment and follow-up, around one fifth of patients with localized bladder cancer will present with disease progression. Adequate prognostic biomarkers are lacking to define patients who are at risk. Mutations in chromatin remodeling genes are more frequently found in bladder cancer than in any other solid tumor. However, the prognostic relevance of epigenetic dysregulation has not been established and may offer an opportunity for biomarker discovery.

Methods: Looking for prognostic epigenetic factors, we performed a comprehensive PubMed search using keywords such as "bladder cancer", "chromatin remodeling", "gene methylation" and "epigenetics". We only included studies reporting on the association of epigenetic markers with prognostic outcomes such as recurrence, progression or survival.

Results: Of 1,113 results, 87 studies met the inclusion criteria, which represented a total of 85 epigenetic markers with potential prognostic relevance. No prospective studies were identified. Seventy-three percent (64/87) of the studies involved mixed cohorts of muscle invasive and non-muscle invasive bladder cancer. Promoter methylation of genes with putative prognostic value affected cellular processes such as cell cycle, apoptosis, cell-adhesion or migration, as well as critical pathways such as MAP-kinase or Wnt. Alteration of chromatin regulatory elements suggest a prognostic relevance alterations leading to a predominantly silenced chromatin state.

Conclusions: Promoter methylation of genes with a putative prognostic value in bladder cancer involves cellular processes and pathways commonly altered in carcinogenesis. Chromatin remodeling alterations leading to a predominantly repressive chromatin state might have prognostic implications. Prospective and mechanistic validation of these findings is required.

Introduction

Bladder cancer is the fifth most common cancer type in both sexes and is more frequent in men, with an estimated 60,490 new cases and 12,240 deaths expected for 2017 in the US alone[1]. Its clinical behavior is usually indolent and, in roughly 70% of cases, presents as a superficial, non-muscle invasive (NMI) tumor that can be cured by transurethral resection (TUR)[2,3]. However, even with adequate treatment and follow-up, progression to muscle-invasive (MI) disease occurs in approximately 21% of patients with high grade disease, which carries a significantly worse prognosis despite aggressive surgical and systemic treatment[3,4]. Thus, it is of utmost interest to identify predictive biomarkers of tumor recurrence and/or progression to help guide clinicians to find optimal treatment strategies.

In bladder cancer, the biological relevance of the hypermethylation of tumor suppressor gene promoters has been widely studied[5,6]. Many investigators have tried to develop gene promoter methylation panels for urine samples as a non-invasive diagnostic method[7]. Also, a large case-control study addressed the potential role of decreased global cytosine methylation in leukocyte DNA and bladder cancer susceptibility[8]. In addition to methylation marks, some studies have assessed the relevance of other genes and proteins with epigenetic regulatory functions, such as chromatin-remodeling genes or non-coding RNAs. Interestingly, chromatin-remodeling gene mutations are highly prevalent in bladder cancer[9–12], which suggests that epigenetic dysregulation is a relevant feature of bladder cancer.

In this article, our aim was to study the clinical relevance of epigenetic dysregulation in bladder cancer. For that, we performed a systematic search of the PubMed database, focusing on the prognostic value of specific epigenetic markers in bladder cancer patients.

Materials and Methods

We performed a systematic literature search of articles published in PubMed up to March 30th 2017. Keywords included "bladder cancer", "transitional cell carcinoma", "chromatin remodeling", "gene methylation" and "epigenetics". We selected those studies that evaluated specific epigenetic marks such as promoter hypermethylation of specific genes and histone tail modifications. We also included studies that evaluated chromatin-remodeling gene alterations and other molecules with a putative epigenetic function, such as transposable elements and non-coding RNAs. Two reviewers (DC and AK) independently screened the abstracts and retrieved full article texts when necessary. Article references were searched to identify additional studies of interest. Studies that evaluated unspecific markers, such as nuclear chromatin shape or global methylation patterns, were excluded. Also excluded were studies that only described the prevalence of the marker(s) of interest and its or their association with other clinical or pathological variables (e.g. tumor stage or grade). Conflicts were resolved by consensus. For each study, basic information including first author name, country and year of publication was extracted and recorded. Additional relevant information included patient number, disease stage, tumor histology, and treatment (Supplementary Table 4). Outcome measures were recurrence and progression rates, recurrence-free and progression-free survival, disease-specific survival, and overall survival. Only markers with relevant prognostic value in at least one study are described in the main text. The detailed search strategy and study exclusion criteria are described in Supplementary Tables 1 and 2. Statistical associations between variables relates to univariate analyses unless stated otherwise in the text, and those corresponding to multivariate analyses are specifically described. The results are organized by pathway or biological function of the markers, which was defined using KEGG, REACTOME and BioSystems annotations.

Results

We included a total of 87 retrospective studies, published between 2001 and 2017 (Figure 1). 26.4% (23/87) of studies involved exclusively non-muscle-invasive bladder cancer (NMIBC) cases and the remaining studies included mixed NMIBC and muscle-invasive bladder cancer (MIBC) patients (Figure 2). Overall, pathological samples were obtained either by transurethral resection (TUR) or radical cystectomy (RC), and studies including upper-tract urothelial carcinoma (UTUC) cases also included nephroureterectomy samples. Gene methylation was generally measured using methylation-specific polymerase chain reaction (MSP), although some studies also included Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) gene methylation panels or direct pyrosequencing of bisulfite-treated DNA samples. More recent studies employed also Methylated DNA immunoprecipitation (MeDIP) or chromatin immunoprecipitation (ChIP) (Supplementary Figure 1).

Some investigators have made a substantial contribution to multiple publications included in this review. In Europe, nine of the thirteen Spanish studies included in this review were led by Dr. Sánchez-Carbayo from CNIO (Spanish National Cancer Research Centre) in Madrid[13–21], of which four were focused on NMIBC [13–16]. Secondly, four out of five British studies were led by Dr. James Catto, from the University of Sheffield[22–25]. Thirdly, six of the nine German studies have been published by Dr. Kurt Miller's team at Charité-Universitätsmedizin Berlin[26–31]. Finally, all of the Portuguese studies were conducted by Dr. Carmen Jerónimo's group in IPO (Portuguese Oncology Institute)[32–36]. Regarding Asian studies, significant contributions have been made by Dr. Ying-Li Lin[37–43] at Jiangsu University and Dr. Liqun Zhou (especially in UTUC studies)[44–46] at Peking University in China, as well as by Dr. Wun-Jae Kim at Chungbuk National University in South Korea[47–54].

1. Gene promoter methylation

1.1. Cell-cycle genes

The Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*) gene encodes two structurally unrelated protein products, p16 and p14[55]. Methylation of *p14* was associated with poor overall survival (OS) in Japanese patients ($p=0.029$)[56]. A study by Dominguez et al involving a Spanish mixed population cohort found higher recurrence rates (RR) for those patients whose tumors harbored either *p16* promoter hypermethylation alone ($p=0.001$) or concomitantly with *p14* promoter methylation ($p=0.01$)[57]. Furthermore, UTUC patients with pTa disease and *p16* methylation had an increased risk of progression ($p=0.03$) in one of the UoS studies[22]. However, many other investigations failed to find a significant prognostic value for *p16/p14* promoter methylation in bladder cancer[13,14,25,41,58–65].

RB promoter methylation had different effects in Spanish NMIBC patients, being associated with a higher risk of recurrence in one study (where *CHFR* methylation was also an adverse factor for recurrence)[13], but protective for progression free survival (PFS) in another ($p<0.05$ for both)[14]. In another CNIO NMIBC cohort, Polyamine-modulated factor 1 (*PMF1*) methylation was associated with better recurrence free survival (RFS) and PFS ($p=0.04$ and $p=0.02$, respectively)[15].

1.2. Cell-adhesion

E-cadherin (*CDH1*) methylation has been reported as predictive of poor PFS (MA: $p=0.02$)[25] and overall survival (OS) (MA: $p=0.02$)[66]. However, *CDH1* methylation was associated with lower progression rates ($p=0.49$) in the first study published by Dr. Catto[22]. Moreover, others have failed to report a prognostic value for this marker [44–46,60,67].

The studies by Dr. Lin at JU have reported that methylation of *CDH13* is associated with increased recurrence risk ($p=0.0043$), shorter time to progression ($p=0.006$), lower RFS ($p<0.0001$)[43] and with an increased risk of death ($p=0.0071$)[41]. In another of his studies, *CDH11* methylation correlated with poor OS ($p=0.0004$)[40]. However, *CDH13* methylation had no prognostic value in other cohorts[13,66]. Dr. Lin's group also reported that protocadherin genes *PCDH17* and *PCDH10* methylation are strong predictors of poor OS ($p<0.05$ for both)[37,42]. Furthermore, he reported that methylation of another protocadherin gene, *PCDH8*, predicted poor RFS in both NMIBC and mixed population studies[38,39].

Dr. Sánchez-Carbayo's group reported lower progression rates for patients with *THBS1* methylation alone or combined with that of other genes such as *RB1*, *TP73* or *MSH6* ($p < 0.05$ for all statistical associations) in NMIBC[14]. On the other hand, *THBS1* methylation showed no prognostic value in the three Chinese UTUC studies conducted by Dr. Zhou [44–46].

In another study by Dr. Sánchez-Carbayo involving NMIBC patients, methylation of myopodin (*SYNPO2*) was associated with increased disease recurrence and progression, as well as with poor DSS ($p < 0.05$ for all correlations) after intravesical adjuvant treatment[16]. In a subsequent mixed population study by the same group, however, *SYNPO2* methylation by itself was not predictive of poor OS ($p = 0.06$)[21]. Finally, Dr. Jerónimo's group reported that methylation of the cell adhesion gene *OPCML* was associated with poor DSS in MIBC patients (MA: $p = 0.003$)[35].

1.3. ECM degradation / Invasion

TIMP3 methylation has been correlated with better RFS in German ($p = 0.036$)[59] and U.S. ($p = 0.039$)[68] NMIBC patients. One of Dr. Carbayo's mixed population studies reported an association between *TIMP3* methylation and poor OS ($p = 0.032$) and CSS ($p = 0.007$)[19]. However, other investigators did not find a prognostic value for this marker[25,58].

VIM methylation has been associated with increased risk of contralateral recurrence ($p = 0.025$)[46], but with better DSS ($p = 0.01$)[33] in UTUC patients. In contrast, in similar studies involving UTUC[44,45] or mixed bladder cancer cohorts[34], *VIM* methylation was not prognostic. Finally, the methylation of extracellular matrix component *LAMC2* was associated with poor OS ($p = 0.03$) in a U.S. cohort[69].

1.4. Apoptosis / p53 pathway

DAPK1 methylation was associated with higher recurrence rates in Japanese NMIBC patients ($p < 0.001$)[60] and also in three mixed population studies ($p < 0.05$)[22,27,70]. However, several studies assessing *DAPK1* methylation have failed to report a prognostic role of this marker[25,26,58,59,63,65].

APAF1 and *IGFBP3* are considered downstream targets of p53. Their methylation has been reported as an adverse prognostic factor for bladder cancer recurrence in four studies conducted by Dr. Miller's group in Berlin[26–28,30]. *IGFBP3* methylation, however, harbored no prognostic value in one of the studies conducted by Dr. Catto's team[25].

PYCARD methylation was associated with high recurrence rates (HR 1.5; $p=0.016$) in one of Dr. Carbayo's NMIBC studies[13]. In that same study, however, this marker held a protective effect for disease progression (HR 0.36, $p=0.006$), DSS (HR 0.295, $p=0.003$) and OS (HR 0.54, $p=0.008$)[13]. In a further study by the same group, methylation of *PYCARD* was also predictive of lower disease progression ($p<0.05$) after intravesical adjuvant treatment[14]. *TP73* methylation was also protective for PFS in that study[14], but was not prognostic in other NMIBC cohorts[13,58].

Methylation of *GDF15* was associated with lower intravesical recurrence rates ($p<0.05$)[44,45] but not with contralateral recurrence[46] in Chinese UTUC patients. However, it was associated with higher cancer-specific mortality in one of these studies ($p=0.002$)[44]. Finally, *GDF15* methylation was not prognostic in one of Dr. Jeronimo's mixed cohort studies[34].

HIC1 methylation, in combination with methylation of *GSTP1* and *RASSF1A*, was associated with better RFS in an Italian NMIBC cohort($p=0.0019$)[58]. In contrast, *HIC1* methylation was not prognostic in one of the studies by Dr. Catto's group[25]. Finally, methylation of *FHIT* was associated with poor OS ($p=0.04$) in a mixed U.S. cohort[66].

1.5. DNA transcription factors

Methylation of the *HOXA9* and/or the *ISL1* genes was found to correlate with higher recurrence rates and poor PFS in Korean NMIBC patients[51] and with higher recurrence, progression and cancer-specific mortality in a British mixed population[71] ($p<0.05$ for all correlations). Of note, a recent study that explored the methylation of the *HOXA* gene locus using MeDIP reported that methylation of the posterior *HOXA* region, as opposed to anterior-only or pan-*HOXA* methylation, was predictive of improved DSS ($p=0.000087$) [72].

RUNX3 has been investigated in several studies led by Dr. Wun-Jae Kim. In Korean NMIBC patients, *RUNX3* methylation has been associated with poor PFS[48,49] and disease recurrence ($p<0.05$ for all associations[49]. In further mixed cohort studies, *RUNX3* methylation has also been associated with increased recurrence rates[50], increased progression rates[50][52] and poor patient survival ($p<0.05$ for all correlations[52][47]. The same investigators reported a robust association of this marker with progression and poor CSS in a further study (MA: $p=0.048$ and $p=0.047$, respectively)[52]. A more recent study including stage IV patients found a strong correlation between *RUNX3* methylation and OS, which

was independent of chemotherapy treatment in MA ($p=0.017$). However, *RUNX3* methylation had no prognostic value in one of the mixed cohort Spanish studies led by Dr. Sánchez-Carbayo [19].

Several other transcription factors have been explored in different clinical settings. *GATA2* methylation was correlated with poor PFS in Dutch patients in two studies published by the same group ($p<0.05$ in both studies)[73,74]. In a Chinese study focusing on NMIBC patients, the hypermethylation of *KLF4* was associated with lower RFS ($p=0.012$) and early recurrence ($p=0.014$)[75]. *SOX9* methylation showed a significant association with poor OS in a Spanish mixed population study ($p=0.025$)[20]. *EOMES* methylation was associated with progression ($p=0.041$, HR=3.79) in a South Korean cohort of NMIBC patients[51]. Finally, *RARB* methylation has been widely studied[13,14,19,22,58,65,68,76], being associated with lower progression rates only in the pT1 patient subset disease of a mixed cohort study ($p=0.001$)[22]. Finally, in a mixed cohort of Taiwanese patients, *ZNF671* methylation was an independent risk factor for predicting recurrence in MA ($p<0.01$) [77].

1.6. DNA repair genes

DNA repair genes have been mainly studied in NMIBC disease. One of the Spanish studies led by Dr. Sánchez-Carbayo found an association between *BRCA1* methylation and lower tumor recurrence rates ($p=0.02$)[13]. However, this finding was not confirmed in the high grade NMIBC cohort published by the same group[14], nor in a similar Italian NMIBC cohort[58]. Of the three Chinese UTUC studies[44–46], *BRCA1* methylation correlated with better CSS ($p=0.04$) only in one study[45]. In contrast, the methylation of *ATM* correlated with higher recurrence rates (MA: $p=0.004$) in the above mentioned Spanish NMIBC cohort[13].

MSH6 methylation correlated with better PFS in high grade NMIBC patients ($p=0.04$)[14] but was not reported as prognostic in a low grade NMIBC cohort analyzed by the same authors[13]. *MLH1* methylation was associated with higher RR in the pTa subset in the study by Catto et al ($p=0.007$)[22]. However, it was not prognostic in two other NMIBC studies [58,60]. Finally, the methylation of *MGMT* has been associated with increased recurrence and progression in NMIBC ($p<0.001$) cohorts [13,78]. However, most of the studies evaluating *MGMT* do not report a prognostic value for this marker [14,25,60,61,63].

1.7. Cell metabolism

GST hypermethylation was associated with increased PFS ($p<0.001$) and OS ($p=0.028$) in the Spanish low-grade NMIBC cohort[13]. In Italian NMIBC patients, *GSTP1* methylation was significantly more frequent in non-recurrent than recurrent cases ($p=0.02$)[58]. In mixed population studies, however, this marker has not shown any prognostic value [22,62]. *ABCC6* methylation has only been studied in Chinese UTUC patients[44,45]. It was associated with higher cancer-specific mortality in one study ($p=0.005$)[44] but was not prognostic in the other[45]. Finally, *ALDH1A3* methylation has been associated with higher recurrence and progression rates in only one study involving Korean NMIBC patients (MA: $p<0.05$ for both)[51].

1.8. Mitogen-activated protein kinase (MAPK) pathway

RASSF1A methylation has been associated with lower recurrences in NMIBC studies ($p<0.05$)[44,58]. Moreover, two previously mentioned Chinese studies conducted in UTUC patients also found lower recurrences for *RASSF1A* methylation ($p<0.05$)[45,46]. This association was also reported in a British cohort including MIBC cases ($p=0.0038$)[62]. In contrast, three studies reported *RASSF1A* methylation to be associated with higher disease progression rates ($p<0.05$)[22,25,79]. An additional study from Pakistan reported shorter OS in *RASSF1A* methylated tumors ($p=0.01$)[61]. However, this marker had no prognostic value in several other studies[59,63,65–67].

The promoter methylation of *TMEFF2* was significantly associated with worse cancer-specific survival (CSS) ($p=0.002$) in Chinese UTUC patients[45]. However, this marker was not prognostic in two other UTUC studies by the same authors[44,46] nor in a Portuguese mixed population study[34].

The methylation of *CACNA1G* was associated with higher recurrence and progression rates, as well as with poorer CSS ($p<0.05$ for all associations) in a mixed population study[19]. Moreover, Catto et al reported with worse PFS for patients whose tumors harbored *MINT31* methylation ($p=0.0081$), a region that codifies for *CACNA1G*[22,80]. Finally, *KISS1* methylation was associated with poor DSS ($p=0.008$) in a mixed cohort study led by Dr. Sánchez-Carbayo[18].

1.9. Wnt-Pathway

APC reached statistical significance as a predictor of poor PFS ($p=0.002$) in one of the studies led by Catto et al[25] and predicted poor OS in a mixed Pakistani cohort ($p=0.03$)[61]. Conversely, *APC* was not prognostic in two other similar studies [58,66].

Methylation of T-box genes *TBX2* and *TBX3* predicted poor PFS in Dutch NMIBC patients[73]. However, in a larger validation cohort ($n=192$) published by the same authors, only *TBX3* methylation was associated with poor PFS in multivariate analysis ($p<0.001$)[74]. In one of the NMIBC studies led by Dr. Sánchez-Carbayo, methylation of Paired Box genes *PAX5A* and *PAX6* was predictive of tumor recurrence ($p=0.01$ and $p=0.04$ for each, respectively)[13]. Recently, however, *PAX6* had no prognostic value in a mixed Canadian population[76].

Methylation of secreted frizzled receptor protein (SFRP) *SFRP5* was associated with disease recurrence ($p=0.001$) and CSS ($p=0.005$) in a mixed cohort study led by Sánchez-Carbayo[19] and was also an adverse prognostic factor in a British mixed cohort study[81]. The prognostic value of the methylation of other SFRP genes has shown inconsistent results[19,81,82].

1.10. Other putative tumor suppressor genes

Methylation of *VHL* or *WT1* was associated with increased disease recurrence in Spanish patients with low grade pT1 disease ($p=0.034$ and $p=0.042$, respectively)[13]. However, *VHL* methylation was not prognostic in a similar Japanese NMIBC cohort[60]. Methylation of *PRAC* independently predicted recurrence and progression ($p=0.012$ and $p=0.035$, respectively) in Korean NMIBC patients[53]. Also in NMIBC, methylation of *ZIC4* has been found to predict poor PFS ($p=0.037$)[74].

2. Chromatin-remodeling complex alterations

In a study by Balbás-Martínez and collaborators, high ARID1A protein expression was associated with poor RFS ($p=0.01$)[83]. Similarly, high ARID1A staining was associated with poor OS ($p=0.048$) in U.S. MIBC patients[84]. Moreover, low expression of PBRM1, another SWI/SNF component, was associated with poor OS ($p=0.007$) and early metastasis onset ($p=0.032$) in Chinese patients[85]. In a study led by

Dr. Miller's group in Berlin, high EZH2 RNA was associated with poor RFS and DSS ($p=0.028$ and $p=0.021$, respectively)[31]. However, EZH2 or other polycomb group components BMI1, SUZ12, RING1, and CBX7 had a prognostic value in other studies by the same group[29,30].

Absence of immunohistochemical expression of the demethylase KDM6A was associated with good PFS ($p=0.049$) in a mixed Danish cohort[86]. In contrast, the same study reported that the overexpression of SMYD3 and EP300 was associated with poor PFS ($p<0.05$ for both) and that of ASH1L with poor DSS ($p=0.0095$)[86]. A further study led by Dr. Jerónimo's group in Portugal found a significant association between high HDAC3 mRNA levels and improved DSS (HR=0.40, $p=0.035$)[32].

WDR5 is part of the MLL/SET1 methyltransferase complex[87]. WDR5 overexpression by immunohistochemistry correlated significantly with poor OS in a Chinese cohort ($p=0.04$)[88]. Furthermore, a German study reported a correlation between MMSET expression and progression ($p=0.00063$)[89]. A further Taiwanese study showed that expression of the methyltransferase DNMT1 by immunohistochemistry was significantly associated with lower responses to concurrent chemoradiotherapy ($p=0.0014$) and reduced survival rates ($p=0.001$)[90]. However, DNMT1 was not prognostic in two other studies conducted in Chinese patients[91,92]. Finally, a study found low expression of the methyltransferase DNMT3b to be significantly associated with poor DFS in a U.S. cohort ($p=0.013$)[93].

3. Long non-coding RNAs and transposable elements

Non-coding RNAs can modulate gene expression by interacting with chromatin remodeling complexes[94,95]. Several studies have assessed the prognostic relevance of long-noncoding RNA (lncRNA) in bladder cancer through microarray screening approaches. The group led by Dr. Catto identified 2075 differentially expressed lncRNAs in a cohort of 57 British bladder cancer samples compared with their paired normal urothelium. Thirty-two of the differentially expressed lncRNAs (see Supplementary Table 3) correlated significantly with tumor progression in a validation cohort of 138 patients ($p<0.05$)[24]. Using a similar approach, He and colleagues identified 85 differentially expressed long intervening non-coding RNA (lincRNA), of which they selected one sequence (termed lincRNA-UBC1) based on its high overexpression in a discovery set. LincRNA-UBC1 was overexpressed in 60 of 102 patients and predicted poor metastasis-free and overall survival ($p<0.05$ for both)[96]. Recently, the same team published a similar analysis in which overexpression of another lncRNA (named "upregulated

in NMIBC” - lncRNAUNMIBC) could independently predict recurrence (HR 2.4, $p=0.007$)[97]. In another Chinese NMIBC cohort, lncRNA HOTAIR overexpression was also identified as a risk factor for recurrence (HR 4.72; $p<0.001$)[98].

Long Interspaced Elements (LINEs) are retrotransposons that regulate gene function by affecting mRNA processing and chromatin structure[99]. A German study found an association between LINE-1 hypomethylation and longer recurrence-free and tumor-specific survival ($p<0.05$)[100].

4. microRNAs

The prognostic value of different micro-RNAs (miR) in bladder cancer has also been investigated. A Danish study focused on the miR-200 family of miRs and reported that low miR-200c expression was correlated with tumor progression in NMIBC ($p<0.01$)[101]. A further study involving Spanish patients identified miR-200a and miR-200b (but not miR200c) overexpression to be associated with better RFS ($p=0.048$ and $p=0.041$, respectively). In the same study, however, they report a correlation between miR-200c overexpression and better OS in 251 MIBC patients from the TCGA dataset ($p=0.015$)[102]. A British study analyzed the methylation of several miRs and their nearby CpG islands and shores and identified miR-1224 CpG shore hypermethylation as a predictor of poor PFS (OR 2.5, $p=0.006$)[23].

5. Histone tail modifications

Global levels of histone methylation marks, such as H4K20 or H3K27 have been investigated. Their methylation levels are reported to be lower in advanced tumors and to be linked to poor overall survival, especially in patients treated with cystectomy[103,104]. In the MIBC subset of a mixed cohort of German patients, increased levels of H4K20me3 were significantly correlated with worse CSS (MA $p=0.006$)[104]. Furthermore, a Chinese study found that high H3K27me3 levels correlated with worse cancer-specific survival after cystectomy, with the only exception being pT4 and pN+ patients ($p<0.05$ for T1-3 and pN- patients)[103]. Finally, in one of the mixed cohort studies led by Dr. Sánchez-Carbayo, *H2AFX1* gene methylation levels were associated with increased recurrence rates in multivariate analysis (HR= 38.934, $p=0.003$)[19].

Discussion

This is, to our knowledge, the largest review on epigenetic alterations with prognostic relevance in bladder cancer. However, there are several limitations to our findings that prevent us from deriving solid conclusions. First, most of the studies included mixed cohorts of NMIBC and MIBC patients and analyzed the markers of interest independently of tumor stage or grade. Second, gene methylation has been assessed by a variety of techniques (Supplementary Figure 1) and authors have employed different (in some cases arbitrary) cut-offs and thresholds of methylation positivity in their studies (Supplementary Table 4), making their results difficult to compare. Third, although it is generally accepted that gene promoter methylation leads to decreased gene expression levels, this is not always true[51], and less than half of included studies evaluated gene expression concomitantly with its methylation (Supplementary Figure 1). Fourth, only a few studies employed a discovery approach, while the vast majority of reports focused on exploratory analyses based on candidate markers. Moreover, very few studies included validation cohorts. Fifth, prognostic assessment was always retrospective and less than half of the studies report detailed patient follow-up. Of note, some studies have been published by the same authors, with very little differences between publication dates, which probably entail population overlaps[15,16,27–29,31,44–46]. All this can lead to a decrease of the biological relevance of the results, and increases publication and selection biases. Therefore, conclusions about tumor biology must be made with caution. These limitations notwithstanding, our findings deserve consideration and may offer some insight for future investigation in bladder cancer epigenetics.

In relevant reports included in our review, we found that gene promoter methylation affected essential components of cancer-related cellular process such as cell-cycle, apoptosis, cell adhesion or migration. Moreover, some of methylated genes are components of relevant signaling pathways in cancer, such as MAPK and Wnt. Some of these pathways and cellular processes have also shown to be altered at the genomic level[9–12]. For example, *CDKN2A* is a common target for loss of heterozygosity in bladder cancer and is commonly accepted as an early event in urothelial carcinogenesis[105,106]. *CDKN2A* deletion was reported in 47% of the TCGA cohort[11] and has recently been linked to poor RFS and CSS in patients with high grade urothelial carcinoma[12]. In our review, *CDKN2A* methylation was reported in up to 64% of bladder cancers. However, the association of this marker with patient outcome was not consistent among studies. Other common targets of deletion in bladder cancer are *RB1* and *FHIT*[11,107], which were also reported as methylated in several studies included in our review.

A striking finding was that several methylated genes apparently showed ambivalent results. For example, methylation of *RASSF1A*, *GDF15* or *TIMP3* promoters was associated with a lower risk of disease recurrence, while remaining adverse prognostic factors for disease progression and/or cancer-specific mortality[19,22,25,44,45,58,59,68]. In some cases, like *RASSF1A*, these observations were overall consistent among studies[22,25,44,58]. Different biological disease pathways could underlie this seemingly paradoxical phenomenon, as happens for some known genetic features that predict indolent or aggressive bladder cancer, such as *FGFR3* mutation[12,108,109]. Mutation of *FGFR3* is generally considered a feature of good prognosis in bladder cancer, defining a subclass of tumors with high recurrence but low progression rates [107]. However, *FGFR3* mutations can also appear in a poor-prognosis context, such as background *CDKN2A* loss [109]. In this context, differences in genomic or epigenomic backgrounds could explain the prognostic ambivalence of the aforementioned methylation marks. Another reason for this observation could be a biased statistical analysis, in which patients that present progression or death events are not computed as events for recurrence.

Chromatin remodeling gene mutations are more frequent in bladder cancer than in any other solid tumor[9,11]. Overall, none of these markers showed a consistent prognostic value in the studies included in this review. Notably, two studies included in our review suggest that overexpression of “closed chromatin” markers predict a favorable prognosis[32,86,103]. Also, a recent study revealed that the permissive chromatin mark H3Ac was decreased in MBIC compared with NMIBC and normal urothelium[110]. If this were confirmed, it would be consistent with current genomic studies, since for example *KDM6A* alterations have been shown to be mutually exclusive with *KMT2D* alterations, suggesting a convergence towards a predominantly silenced chromatin during bladder carcinogenesis[11,12,107]. Finally, although only few studies evaluated the prognostic relevance of non-coding RNAs, their overexpression was generally considered an adverse prognostic factor. Interestingly, non-coding RNAs such as HOTAIR, lnc-UBC1 and the miR-200 family have been reported to interact with repressive complexes [94,96,102,111]. Therefore, further studies are needed to validate the prognostic relevance of chromatin regulatory elements in bladder cancer.

Conclusions and future perspectives

Two main epigenetic alterations with potential prognostic value have been reported in bladder cancer: changes in DNA methylation and alterations in epigenetic regulatory elements. Notably, although the two phenomena have been usually studied and reported separately, they probably represent different aspects of the same continuum. The exact mechanisms that connect regulatory elements with the different methylation patterns and their clinical relevance are still to be defined. Fortunately, new methodologies that allow for reliable analyses of chromatin patterns in FFPE-preserved tumor tissues will help us understand more complex chromatin-organization patterns and help us unveil critical regulators of gene expression[112]. In future studies, epigenetic and genetic will need to be integrated in order to improve our molecular understanding of bladder cancer. Perhaps, epigenetic markers such as *RASSF1A* or *CDKN2A* methylation could add information to the currently existing molecular classification of bladder cancer[113].

Epigenetic alterations open therapeutic opportunities for urothelial tumors[114,115]. Some preclinical data have suggested that tumors harboring inactivating mutations or homozygous deletions in CREBBP and/or EP300 may be sensitive to HDAC inhibition[116]. Also, several small molecular inhibitors against chromatin-regulating proteins have shown potential for the treatment of urothelial tumors[117]. Several clinical trials are underway using the epigenetic alterations as targets for therapeutic intervention[118]. A phase II study of the HDAC inhibitor Mocetinostat in patients with urothelial carcinoma selected for inactivating alterations of acetyltransferase genes (NCT02236195) has recently completed accrual and results are eagerly awaited.

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Figure 1

Figure 1. Study Flow Chart

Figure 2

Figure 1. Characteristics of the included studies.
Abbreviations: NMIBC non-muscle invasive bladder cancer, MIBC muscle invasive bladder cancer

Table 1

Pathway	Gene(s)	Prognostic		Not Prognostic		Pathway	Gene(s)	Prognostic		Not Prognostic	
		<u>NMIBC studies</u>	<u>Mixed population studies°</u>	<u>NMIBC studies</u>	<u>Mixed population studies°</u>			<u>NMIBC studies</u>	<u>Mixed population studies°</u>	<u>NMIBC studies</u>	<u>Mixed population studies°</u>
<u>Cell cycle</u>											
	<i>CDKN2A</i>		[22,56,57,67]	[13,14,58–60,65]	[25,61–63,41,64]		<i>ISL1</i>	[51]	[71]		
	<i>RB1</i>	[13,14]					<i>SOX9</i>		[20]		
	<i>CHFR</i>	[13]				<u>DNA repair</u>					
	<i>PMF1</i>		[15]				<i>BRCA1</i>	[13]	[45]	[14,44,58]	[46]
<u>Cell adhesion</u>											
	<i>CDH1</i>		[22,25,66]	[44,60]	[45,46,67]		<i>MLH1</i>			[22]	[58,60]
	<i>CDH13</i>	[43]	[41]	[13]	[66]		<i>MGMT</i>	[13,78]		[14,60]	[25,61,63]
	<i>THBS1</i>	[14]		[44]	[45,46]		<i>MSH6</i>	[14]		[13]	
	<i>PCDH8</i>	[38]	[39]			<u>Cell metabolism</u>					
	<i>PCDH17</i>	[37]					<i>GSTP1</i>	[13,58]			[22,62]
	<i>SYNPO2</i>	[16]			[21]		<i>ABCC6</i>	[44]			[46]
	<i>CDH11</i>		[40]				<i>ALDH1A3</i>	[51]			
	<i>PCDH10</i>		[42]			<u>MAPK signaling</u>					
	<i>OPCML</i>		[35]				<i>RASSF1A</i>	[44,58]	[22,25,45,46,61,62,79]	[59,65]	[63,66,67]
<u>ECM degradation/ Invasion</u>											
	<i>TIMP3</i>	[59,68]	[19]	[58]	[25]		<i>TMEFF2</i>		[45]	[44]	[34,46]
	<i>VIM</i>		[33,46]	[44]	[34,45]		<i>CACNA1G</i>		[19,22]		
	<i>LAMC2</i>		[69]				<i>KISS1</i>		[18]		
<u>Apoptosis / p53 pathway</u>											
						<u>Wnt pathway</u>					
							<i>APC</i>		[25,61]	[58]	[66]

DAPK	[60]	[22,27,70]	[58,59,65]	[25,26,63]	SFRP1	[81]	[82]
APAF1		[26–28,30]			SFRP2	[81]	
					SFRP3		
GDF15	[45]	[44]		[34,46]	SFRP4	[81]	[19]
IGFBP3		[26,28,30]		[25]	SFRP5	[19,81]	
TP73	[14]		[13,58]		TBX2	[73,74]	
HIC1	[58]			[25]	TBX3	[73,74]	
PYCARD	[13,14]				PAX5A	[13]	
FHIT		[66]			PAX6	[13]	[76]
DNA transcription					Other		
RARB	[65]	[22]	[13,14,58,68]	[19,76]	PRAC1	[53]	
RUNX3	[48,49]	[47,50,52]		[19]	WT1	[13]	
HOXA9	[51]	[71,72]			ZIC4	[74]	
EOMES	[51]				VHL	[13]	[60]
KLF4	[75]						
GATA2	[73,74]						
ZNF671		[77]					

TABLE 1. Methylation marks with prognostic relevance in bladder cancer.

°Mixed population studies refer to those studies that included mixed cohorts of patients with non-muscle invasive and muscle invasive bladder cancer

Abbreviations: NMIBC non-muscle invasive bladder cancer, MIBC muscle-invasive bladder cancer, MAPK mitogen-activated protein kinase, ncRNA non-coding RNA, TSG tumor suppressor gene, ECM extracellular matrix

Gene abbreviations: ABCC6 ATP Binding Cassette Subfamily C Member 6, ALDH1A3 Aldehyde dehydrogenase 1 family member A3, APAF-1 Apoptotic Peptidase Activating Factor 1, APC Adenomatous Polyposis Coli, BRCA1 breast cancer type 1 susceptibility gene, CACNA1G Calcium Voltage-Gated Channel Subunit Alpha1 G, CDH1 cadherin 1 or E-Cadherin, CDH11 cadherin 11, CDH13 cadherin 13, CDKN2A cyclin-Dependent Kinase Inhibitor 2A, DAPK death-associated protein kinase 1, EOMES eomesodermin, FHIT fragile Histidine Triade, GATA2 GATA binding protein 2, GDF15 growth differentiation factor-15, GSTP1 glutathione S-Transferase Pi 1, HIC1 Hypermethylated in Cancer 1, HOXA9 Homeobox A9, IGFBP3 Insulin-Like Growth Factor-Binding Protein 3, ISL1 ISL LIM homeobox 1, KISS1 KiSS-1 Metastasis-Suppressor, KLF4 Kruppel Like Factor 4, LAMC2 Laminin Gamma 2 chain, MGMT O-6-Methylguanine-DNA Methyltransferase, MSH6 MutS Homolog 6, OPCML Opioid Binding Protein/Cell Adhesion Molecule Like, PCDH10 Protocadherin 10, PCDH17 Protocadherin 17, PCDH8 Protocadherin 8, PRAC1 Prostate Cancer Susceptibility Candidate 1, PYCARD PYD and CARD Domain containing, RARB β Receptor of Retinoic Acid, RASSF1A RAS Association Domain-Containing Protein 1,

RB1 Retinoblastoma 1, *RUNX3* The Runt domain transcription factors, *SFRP* Secreted Frizzled Related Proteins, *SOX9* SRY(sex-determining region Y)-related high-mobility group box 9, *SYNPO2* (Myopodin) Synaptopodin 2, *TBX2* T-Box 2, *TBX3* T-Box 3, *THBS1* Thrombospondin 1, *TIMP3* Tissue inhibitor of Metalloproteinases 3, *TMEFF2* Transmembrane Protein With EGF Like And Two Follistatin Like Domains 2, *TP73* Tumor Protein P73, *VHL* Von Hippel-Lindau Tumor Suppressor, *VIM* Vimentin, *ZNF671* Zinc Finger Protein 671, *PAX5A* Paired Box Homeotic Gene 5, *WT1* Wilms tumor 1, *CHFR* Checkpoint With Forkhead And Ring Finger Domains, E3 Ubiquitin Protein, *ATM* Ataxia Telangiectasia Mutated Serine/Threonine Kinase.

Table 2

Pathway	Gene(s)	Prognostic		Not Prognostic		Pathway	Gene(s)	Prognostic		Not Prognostic	
		<u>NMIBC studies</u>	<u>Mixed population studies</u> ^o	<u>NMIBC studies</u>	<u>Mixed population studies</u> ^o			<u>NMIBC studies</u>	<u>Mixed population studies</u> ^o	<u>NMIBC studies</u>	<u>Mixed population studies</u> ^o
<u>Chromatin remodeling complex alterations</u>						<u>LncRNA and transposable elements</u>					
	RSF1		[119]				32 LncRNAs				[24]
	PBRM1		[85]				LncRNA-UNMIBC	[97]			
	ARID1A		[83,84]				<i>lnc-UBC1</i>				[96]
	EZH2		[31]				<i>HOTAIR</i> ^o	[98]			
	WDR5		[88]				<i>LINE-1</i>	[100]			
	SMYD3		[86]			<u>miRNAs</u>					
	KDM6A		[86]				<i>miR152</i>		[23]		
	EP300		[86]				<i>miR1224</i>		[23]		
	MMSET	[89]					<i>miR200a</i>		[102]		
	DNMT3b		[93]				<i>mir200b</i>		[102]		
	ASH1L		[86]				<i>miR200c</i>		[101,102]		
	DNMT1		[90]			<u>Histone tail modifications</u>					
	HDAC3		[32]				H3K27		[103]		
							H4K20		[104]		
							H2AFX		[19]		

TABLE 2. Regulatory elements with prognostic relevance in bladder cancer.

^oMixed population studies refer to those studies that included mixed cohorts of patients with non-muscle invasive and muscle invasive bladder cancer

Gene abbreviations: ARID1A AT-rich interacting domain containing protein 1A, DNMT1 DNA Methyltransferase 1, DNMT3b DNA Methyltransferase 3 beta, EZH2 enhancer of Zeste homolog 2, H2AFX Histone Family Member X, H4K20me3 trimethylation of lysine 20 on histone 3, HDAC3 Histone Deacetylase 3, HOTAIR HOX Transcript Antisense RNA, PBRM1 Polybromo 1, PMF1 Polyamine Modulated Factor 1, RSF1 Remodeling And Spacing Factor 1, WDR5 WD Repeat Domain 5, lncRNA long non-coding RNA, SMYD3 SMYD family member 3, KDM6A Lysine Demethylase 6A, ASH1 Like Histone Lysine Methyltransfer