Incorporating DNA Methyltransferase Inhibitors (DNMTis) in the Treatment of Genitourinary Malignancies – a systematic review

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Abstract

Inhibition of DNA methyltransferases emerged as a novel treatment strategy in solid tumors. Aberrant hypermethylation in promoters of critical tumor suppressor genes created a premise, that treatment with hypomethylating agents may lead to restoration of "normal" epigenome and produce clinically meaningful therapeutic outcome. The aim of this review article is to summarize the current state of knowledge of DNA methyltransferase inhibitors in the treatment of genitourinary malignancies. We performed a literature search of PubMed/Medline, ASCO, ASCO GU and AACR libraries on September 17th 2017. Included reports evaluated pre-clinical and clinical studies assessing the role of DNA methyltransferase inhibitors in genitourinary malignancies. The efficacy of DNA methyltransferase inhibitors in genitourinary malignancies was reported in number of studies and suggested the role of DNA hypomethylation in overcoming resistance to conventional cytotoxic treatments. The clinical significance should be further investigated.

Key points

Increasing knowledge about epigenetic landscape in cancer leads to discovery of promising novel drugs.

Evidence from pre-clinical and clinical studies suggests that DNA methyltransferase inhibitors provide anticancer activity in number of tumors.

DNA methyltransferase inhibitors deliver cytotoxic and chemoresistance overcoming effects in several genitourinary malignancies.

1. Introduction

The conventional paradigm of phenotype manifesting as a sole result of the information encoded in the DNA experienced radical shift over the recent years. Epigenetic changes represent series of mechanisms interfering with the DNA and its function without altering the sequence in coding genes. Both, genetic and epigenetic aberrations cooperate to produce conformational changes in chromatin and biochemically alter gene promoters. Such regulation induces or represses the transcriptional gene activity and may ultimately lead to carcinogenesis [1, 2].

Recent studies indicate that epigenetic silencing may be as important as DNA mutations in tumorigenesis and treatment resistance [3]. While mutations represent an irreversible change in the DNA sequence, epigenetic silencing is a reversible process. Known modifications with ability to influence the gene expression without altering the DNA sequence include DNA methylation, histone modifications, nucleosome remodeling induced by ATPases, and regulation via non-coding RNAs[3-6]. This review will summarize the current state of pre-clinical and clinical knowledge in targeting DNA methyltransferases, a key messengers for DNA methylation, in genitourinary cancer.

2. Literature search

We performed a literature search of the PubMed/Medline database, and meeting libraries of American Society of Clinical Oncology (ASCO), ASCO Genitourinary Cancers Symposium and

American Association for Cancer Research (AACR) for publications with the terms “epigenetics”, “DNMT”, “DNMTi” “genitourinary”, “testicular cancer”, “germ-cell tumors”, “bladder cancer”, “renal cell carcinoma”, “prostate cancer”, “penile cancer”, “azacitidine”, “decitabine”, “guadecitabine”, “zebularine”, “non-nucleoside”. Combinations of these key words were used for comprehensive search as outlined in Figure 1. The search of literature was performed on September 15th 2017. Original full-text articles published in English were reviewed and the reference lists of key articles were further evaluated. We did not limit our search by the years of publication. Our search was conducted according to the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) statement. Identified reports were reviewed according to the Consolidated Standards of Reporting Trials (CONSORT) criteria. The search resulted in overall 4, 152 publications. One hundred and eleven publications were selected for inclusion in our review article [88 original papers (79%) and 23 (21%) review articles]. The literature search and the inclusion and exclusion criteria are summarized in Figure 1 and Table 1.

3. DNA Methylation

A covalent addition of methyl groups (-CH3) to the carbon-5 position of cytosine represents a non-coding modification of DNA – a methylation [7]. DNA methylation at the 5′-CG-3′ sequence (also CpG dinucleotide) is a unique, heritable mechanism for regulation of gene transcription. Hypermethylation generally acts as gene silencer by downregulating the transcription of CpG-rich promoter regions (CpG islands) of tumor suppressors [8]. 5-methylcytosine may undergo a spontaneous deamination to thymine allowing for changes in the chromatin structure. This represents a change in DNA sequence and creates a significant restraint to initiate a transcriptional activity. DNA methylation may be found in distinct locations that may

further influence gene expression and results in different biological phenotypes. Promoter region methylation is a repressive mark, while the gene body methylation is usually associated with active gene expression [9, 10]. DNA hypermethylation in the promoter region of tumor suppressor genes in human cancer may represent one of the most important mechanisms in cancer development [11].

DNA methylation was discovered to be present also in context of non-CpG (CpA, CpT, CpC). Non-CpG methylation coexists with CpG methylation, but the exact role is not clear. Evidence suggests an involvement in gene expression when non-CpG methylation is found in promoter regions [12]. Unlike CpG methylation, non-CpG methylation is asymmetrical and thus the same methylation pattern after each cell division cannot be maintained [13]. As a result, non-CpG methylation is decreased in rapidly proliferating tissues suggesting a potential role in carcinogenesis and pluripotency [14, 15].

The current discoveries have identified significance of enzymatic activity of three DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B. The role of DNMT1 is to maintain the existing methylation patterns, while DNMT3A and DNMT3B are responsible for *de novo* methylation[16].

4. Proposed mechanism of action of DNA methyltransferase inhibitors (DNMTis) in solid tumors

In-depth understanding of the mechanisms underlying the effects of DNMTis is virtually non-existent. Thus, hypotheses and emerging evidence are outlined in this review article in attempt to summarize the current state of knowledge. A well-proven fact that DNMTis are responsible for global hypomethylation resulting in (re)expression of certain tumor suppressor genes offers a superficial knowledge on how DNMTis deliver their effect [17, 18]. On the other hand, global

hypomethylation in tumors was linked with aberrant activation of cancer germ-line oncogenes that promote cell proliferation, angiogenesis and metastasis [19]. It is unclear, whether the intra-tumoral hypomethylation activating oncogenes can be overcome by treatment effects of DNMTis which alter the whole body methylome. Non-specificity of treatment with DNMTis may raise doubts about the safety of drugs with the potential to induce re-expression of tumor-suppressor genes as well as oncogenes. Available data from a phase II clinical trial in ovarian cancer did not show any secondary malignancies in patients treated with decitabine for 2-30 months [20]. Primary target population for clinical trials evaluating the efficacy of these novel agents are patients with refractory solid tumors pretreated with conventional treatments. Described evidence suggests that survival benefit of treatment with DNMTis in this patient population may outweigh the risk of potential secondary malignancies. The most common toxicities are nausea, constipation, allergic reactions and bone marrow suppression. Grade 3-4 neutropenia and thrombocytopenia were seen in 22% and 11% of patients respectively [20].

Most of the initial clinical trials using a single agent DNMTi to treat solid tumors were unsuccessful in improving outcomes. DNMTis were used at high doses with the intent to deliver a non-specific cytotoxic effect, however, the delivery at low doses are optimized to inhibit DNA methylation [20-22]. More recently, trials are testing whether DNMTis could sensitize refractory tumors to chemotherapy and whether they can improve immune response and boost immune therapy.

4.1 DNMTis to overcome chemo-resistance

Multiple hypotheses are proposed to explain the role of DNMTis in overcoming resistance to chemotherapy, specifically the DNA targeted drugs (DTDs). Lethal DNA damage is the main mechanism of cell death induced by platinum compounds and doxorubicin [23]. Cisplatin induces

DNA breaks by creating inter-strand adducts. Doxorubicin also creates DNA damage after being incorporated into DNA. DNMTis may potentiate the DNA damage induced by cisplatin and doxorubicin by increasing the accessibility of these drugs through loosening chromatin globally, which is required for DTD incorporation and DNA damage [24]. Furthermore, epigenetic synergy of decitabine and platinum agents, but no other cytotoxic drugs, was observed by Qin *et al.* in colon cancer cell line. Treating the cell line with 16 cytotoxic drugs including platinum agents did not result in activation of hypermethylated cytomegalovirus promoter. However, the addition of platinum compounds achieved a striking synergy in activating the promoter. Experiments also resulted into significantly better reactivation of hypermethylated tumor suppressor genes (*MHLM1* and *PDLIM4*) with the combination of decitabine and carboplatin, compared to each drug alone, therefore offering evidence that platinum resistance can be overcome with the addition of decitabine [18]

4.2 DNA methyltransferase (DNMT) inhibitors and immune responses

DNMTis have potency to reactivate the silenced tumor suppressor genes by DNMT inhibition, and are capable of upregulating the genes encoding MHC I molecules, tumor antigens [25-27] and interferon (IFN) response proteins [28]. Decitabine upregulated chemokine expression *in vitro* in ovarian tumor cell and increased the number of NK and CD8+ cells in malignant ascites in an orthotopic mouse model [29].

Recent reports suggest immune modulation as a possible mechanism of action for azacitidine. The expression of endogenous retroviruses (ERVs) is silenced by DNA methylation [30, 31], and treatment with low doses of azacitidine reactivated ERVs in ovarian and colon cancer cells [32, 33]. While most ERVs have lost the ability to mature and infect other cells, they can still expand within their host

genome [27, 34]. Upon the expression of ERV, a double-strand RNA (dsRNA) that is constructed plays a role in creating of a type I IFN response [32, 33]. As a result, azacitidine may induce tumor cells to mimic virally infected cells, resulting in an antitumor immune response [35]. Several clinical trials with azacitidine or guadecitabine in combination with immune checkpoint inhibitors are ongoing in various solid tumors.

5. DNA methyltransferase inhibitors (DNMTis) and their mechanism of action

Historical pre-clinical and clinical studies assessed 5-azacitidine (azacitidine) and 5-aza-2′deoxycytidine (decitabine) that were among the first agents efficient in downregulating the DNA methylation. Azacitidine and decitabine act as inhibitors of DNMTs and are now referred to as "first generation DNMTis" preceding a "second generation DNMTis" guadecitabine (2´-deoxy-5-azacytidylyl-(3´-5´)-2´-deoxyguanosine sodium salt) and zebularine (1-[beta]-D-ribofuranosyl]-1,2-dihydropyrimidin-2-one). These agents act as cytidine antimetabolite analogues creating covalent complexes with DNMTs and DNA. Trapping the DNMTs in this fashion ultimately alters their function resulting in downregulation of DNA methylation [36]. This covalent trapping, however, also induces DNA damage that was suggested in the mutagenic and cytotoxic effects of DNMTis [37]. Azacitidine and decitabine were therefore initially developed as chemotherapeutic agents and were used at cytotoxic doses.

First generation DNMTis suffer from fast metabolism induced by hydrolytic cleavage and deamination. As a result, their stability in the blood is limited and their use as cytotoxic agents in solid cancers proved unsuccessful [38]. The second-generation DNMTis guadecitabine and zebularine are stable in the liquid environment and allow for a longer effective half-life [38-40]. While hypomethylation induced by the first generation DNMTis in *in-vitro* studies [41-43] brings evidence

of effectivity of these agents, downregulating the DNA methylation with guadecitabine in cancer xenograft models [44] provided proof that unfavorable pharmacokinetic profile of first generation DNMTis can be overcome. Zebularine was shown to be preferentially incorporated in zebularine-sensitive tumor cells with specific transcriptomic and epigenomic signature in *in-vitro* and xenograft mouse model experiments with human liver cancer. This shows a promising tool for predicting response to this second generation DNMTi [45]. Fluorocyclo-pentenylcytosine is another novel next generation nucleoside agent with antimetabolic activity that also acts as DNMT1 inhibitor. Numerous preclinical cell-line and xenograft animal models have shown antitumor activity of this drug [46-49].

Alternative mechanism that reduces CpG island methylation is inhibition of DNMT1 with antisense oligonucleotides which are not incorporated into genomic DNA. In-vitro experiments in bladder cancer cells have shown that MG88 targeting the 3'-untranslated region of *DNMT1* resulted in suppressed DNMT1 expression, thus allowing for re-expression of tumor suppressor gene *α-CDKN2A* [17].

Mechanism involved in transport of DNMTis across cell membrane indirectly influence the ability of DNMTis to perfom effectively. Human organic cation and nucleoside transporters may both mediate intake or efflux of azacitidine, decitabine and zebularine. These transporters, thus, contribute to chemoresistance or chemosensitivity to DNMTis in cancer therapy [50].

Several other non-nucleoside targeting DNMTi agents are procaine [51] N-acetylprocainamide, procainamide (perturbing the interactions between the protein and its target sites), hydralazine (decreases the expression of DNMT1 and 3A)[52], (−)-epigallocatechin-3-gallate (EGCG, a catalytic pocket blocker of DNMT1 found in green tea)[53], and RG108 (the first rationally designed inhibitor of DNMTs)[54]. Non-nucleoside agents have shown considerably less demethylating activity in

bladder and prostate cancer cell lines compared to decitabine [52]. Ongoing clinical trials using DNMTi agents are summarized in Table 2.

6. Targeting DNMTs in genitourinary cancer

Following chapters are discussing a current state of evidence in the pre-clinical and clinical use of DNMTis in genitourinary malignancies.

6.1 Germ Cell Tumors

Germ cell tumors (GCTs) have a global DNA hypomethylation that may explain an exceptional sensitivity to platinum-based chemotherapy [55-57] resulting from the highest de novo DNA methyltransferase expression (DNMT3A/3B) among solid cancers (Figure 2) [58, 59]. However, numerous promoters in GCT cell lines were discovered to be hypermethylated in non-seminomas[60] and promoter hypermethylation of *RASSF1A* and *HIC1* genes was linked to cisplatin resistance in embryonal carcinoma cell lines[61]. Beyrouthy *et al.* have shown that overexpression of DNMT3B is associated with hypersensitivity to decitabine. Treatment with decitabine resulted in a re-sensitization of testicular cancer cells to cisplatin. Furthermore, the demethylation resulted in a re-activation of tumor suppressor genes [62]. Similar observations were made by Wermann *et al.*, who observed an increased sensitivity to cisplatin after treatment with 5-azacitidine in platinum resistant GCT cell lines [57].

We evaluated guadecitabine *in vitro* and in an *in vivo* in a mouse model of cisplatin refractory GCT and found that testicular cancer cells had exquisite sensitivity to single agent exposure. Guadecitabine completely abolished progression and induced complete regression of cisplatin resistant testicular cancer xenografts even at doses well below those required to impact somatic

solid tumors [22]. A phase I study of guadecitabine in combination with cisplatin in refractory GCT subjects who relapsed after multiple lines of chemotherapy including high-dose chemotherapy (HDCT) is currently ongoing at Indiana University (NCT02429466). Anecdotal evidence from a phase II study evaluating hydralazine and magnesium valproate showed a stable disease in 1 patient with refractory non-seminoma [63].

6.2 Bladder cancer

Aberrant DNA methylation was initially found to be relevant to carcinogenesis in human bladder tumors and cell lines. Later, comprehensive genomic and promoter assessments have shown characteristic DNA methylation patterns in bladder cancer [64, 65]. Maruyama *et al.* assessed the promoter methylation status of several cancer-related genes including *CDH1, RASSF1A, APC, CDH13, FHIT, RARβ, GSTP1, p16INK4a, DAPK* and *MGMT* in 98 bladder tumors. High methylation frequency in *RASSF1A, APC, CDH1, CDH13* and *FHIT* correlated with poor prognostic clinical-pathological features and shorter overall survival [66]. Hypermethylation of *RASF1A, APC* and *MGMT* has been linked to high grade and invasive tumors in 76 patients with urothelial bladder cancer [67]. In pre-clinical studies, azacitidine inhibited the proliferation of bladder cancer cells and arrested cells at the G0/G1 phase. Wang *et al.* performed *in vivo* and *in vitro* experiments showing that azacitidine markedly downregulated the expression of DNMT3A/3B, reactivated expression of hepaCAM, and inhibited bladder cancer growth in nude mice [68]. Treatment with demethylating agents decitabine and zebularine (nucleoside analog of cytidine) induced a growth inhibition in urothelial bladder cancer and renal cell cancer cells resulting in a 17-132% prolongation of cell doubling time [69]. A preclinical study evaluating single agent azacitidine in 19 dogs with spontaneous urothelial bladder cancer confirmed a

myelosupressive, but relatively safe toxicity profile. Partial remission was seen in 22%, stable disease in 50% and progressive disease in 22% of dogs evaluable for tumor response [70]. Another pre-clinical study showed that sensitization of cisplatin resistant bladder cancer cell lines can be achieved *in vitro* by decitabine pre-treatment alone and in combination with the HDAC inhibitor vorinostat [71]. Chuang *et al.* conducted a pre-clinical *in vivo* assessment of single agent decitabine and guadecitabine in murine xenograft models derived from bladder cancer cell lines. Intraperitoneal delivery of both drugs was effective in reducing the level of DNA methylation at the P16 promoter. Murine tumors also exhibited a growth inhibition, although this treatment was insufficient to reduce the size of the tumors. A subcutaneous administration delivered similar results. Toxicity profile, however, was more favorable with subcutaneous injection [72]. While zebularine effectively induced hypomethylation[73] and reactivated silenced genes[74] in T24 bladder cell lines, another study showed its' complex metabolism and limited incorporation into the DNA in bladder cancer cell lines. This may be the reason for lower efficacy of zebularine compared to azacitidine and decitabine in bladder cancer *in vitro* [75]. Interestingly, zebularine increased radiation-induced DNA damage in bladder cancer cell line and increased the radiation induced tumor growth delay in xenograft mouse model [76]. The function of DNMT1 in bladder cancer cell lines was recently discovered to be mediated by the virtue of long non-coding (lnc) RNA *DBCCR1-003* derived from the locus of *DBCCR1* tumor suppressor gene [77]. Habuchi *et al*. previously linked the loss of *DBCCR1* expression and its association with transitional-cell bladder cancer [78]. Qi *et al*. reported that *DBCCR1-003* normally binds to DNMT1 and prevents the hypermethylation of *DBCCR1* thus allowing its expression. Treatment with decitabine or overexpression of *DBCCR1-003* resulted into increased expression of *DBCCR1* via reversed promoter hypermethylation and DNMT1 binding to *DBCCR1-003* and promoter *DBCCR1* in T24

bladder cancer cell line. Such process led to significant growth inhibition of the cell line suggesting DBCCR1-003 as novel biomarker and potential treatment target [77]. *In-vivo* experiments in patient-derived bladder cancer xenografts in mice showed that guadecitabine reduced DNA methylation at the *p16* promoter region and reduced the growth of tumors in xenografts [79].

A combination of azacitidine with sodium phenylbutyrate was used in a phase I study of patients with refractory solid tumors. Two of 28 patients had bladder cancer, however, no objective responses were observed within this trial [80]. Another phase I study used azacitidine with valproic acid in 55 patients with refractory malignancies of whom 3 had advanced urothelial cancer. The combination did not produce any objective responses. Stable disease was achieved in 25% of patients with various malignancies, other than urothelial carcinoma [81].

Two clinical trials evaluating treatment with decitabine (NCT00030615) and CC-486, oral formulation of azacitidine (NCT01478685) in solid tumors including bladder cancer were conducted, but to our best knowledge, the results were not reported yet. Another clinical study (NCT02223052) with CC-486 in solid tumors including genitourinary cancer is currently underway. Additionally, a genomic-based assignment of treatment including azacitidine and decitabine is currently ongoing in advanced urothelial carcinoma (NCT02788201).

A phase I study of antisense oligonucleotide inhibitor of DNMT1 assessed the safety and efficacy of MG98, given in an infusion over 7 days to 33 patients with solid tumors, including bladder, upper urinary tract and prostate cancer. Treatment related toxicities were generally mild and most common being fatigue, headache and myalgia of grade ≤ 2. Dose limiting toxicities were grade 3 transaminitis and grade 3 thrombocytopenia. Evidence of activity was observed in this study, however, none of the patients that responded had genitourinary cancers [82].

Fluorocyclo-pentenylcytosine (RX-3117) have shown antitumor activity in gemcitabine resistant pre-clinical models and is currently being evaluated in a phase I/II study in pancreatic and urothelial cancer (NCT02030067) [46-49].

The insufficient clinical activity of most of the DNMTis in urothelial cancer could be explained by the short half-life of the first generation DNMTi agents. From a mechanistic standpoint, DNMTis may be better used in combination with cytotoxic drugs such as cisplatin or with immune checkpoint inhibitors.

6.3. Renal Cell Carcinoma

Abnormal hypermethylation as well as hypomethylation of DNA may occur in renal cell carcinoma (RCC), resulting in activation of tumorigenesis and chromosomal instability [83, 84]. Numerous tumor suppressor genes have been reported to be partially or completely silenced due-to hypermethylation of their enhancer and promoters leading to increased tumor cell proliferation, invasion, and metastasis [85]. Deep DNA methylation and transcriptome profiling of diverse histological RCC subtypes uncovered that clear cell (ccRCC), papillary and translocation RCC as well as mucionous and spindle cell carcinomas, are 3-fold more hypermethylated as compared to oncocytoma and chromophobe RCC [86]. Moris *et al.* described nine genes showing frequent promoter region methylation in primary RCC tumor samples. The methylation of SCUBE3 was associated with a significantly increased risk of cancer death or relapse [87]. Li *et al.* found that DNMT1 protein was expressed significantly higher in ccRCC compared to normal tissues (56.2% and 27.3%, respectively). The expression of DNMT1 was positively correlated with tumor size, highly malignant phenotype, lymph node metastasis, vascular invasion, recurrence and poor prognosis. This observation was confirmed *in vitro* in cell lines, where knockdown of DNMT1 significantly inhibited ccRCC cell viability, induced apoptosis, decreased colony formation and

the invading ability [88]. German group recently proposed metastasis associated methylome signature obtained from genome-wide TCGA dataset. Authors predicted metastatic disease with 93% sensitivity and 89% specificity and proposed the prospective validation of this tool [89].

Treatment with azacitidine resulted in suppression of cell proliferation in all 15 RCC cell lines evaluated by Ricketts *et al*. Interestingly, response correlated with alterations in VHL promoter methylation, however some cell lines without VHL tumor suppressor gene methylation responded to treatment as well. This finding is suggestive of other hypermethylated suppressor genes activated with DNMTi treatment with several hypermethylated candidate tumor suppressor genes identified (RGS7, NEFM, TMEM74, GCM2 and AEBP1). Methylation of GCM2, NEFM and RGS7 also strongly correlated with poor prognosis [90]. Treatment of A-498 RCC cell line with low-dose zebularine resulted in limited cell inhibition, however, authors observed an up-regulation and down-regulation of 308 and 253 gene transcripts, respectively. Many of the re-expressed genes belong to metallolthionein family, potent protectors against oxidative stress, that were discovered to be down-regulated in RCC tumors [91]. Decitabine, but not zebularine was able to reexpress a hypermethylated VHL gene in RCC cell lines and caused a tumor shrinkage in RCC xenograft mouse model. Only tumors with hypermethylated VHL gene responded to treatment. VHL mutated mice did not show any response [92]. Guadecitabine showed an interesting potential to increase immunogenicity of renal cell carcinoma and other tumors cell lines. Treatment with guadecitabine induced a de novo expression or reexpression of cancer testis antigen related genes (*MAGE-A1*, *MAGE-A2*, *MAGE-A3*, *MAGE-A4*, *MAGE-A10*, *GAGE 1-2*, *GAGE 1-6*, *NY-ESO-1*, and *SSX 1-5*) and HLA class I antigens of ICAM-1, an important factor for improved recognition of cancer cells by gp-100 specific cytotoxic T lymphocytes [93].

Reu and colleagues have shown a synergistic potential of cytokine therapy combined with decitabine. Resistance to the antiproliferative and apoptotic effects of interferons (IFNs) was postulated to result from silencing of IFN response genes by promoter hypermethylation. Treatment of human RCC cells with decitabine overcame IFN resistance, while normal epithelial kidney cells remained unaffected. IFN response gene expression was augmented greater than 10 times by decitabine [94].

A phase I study conducted by Lin *et al.* aimed to find the safe and effective dose of azacitidine in combination sodium phenylbutyrate in refractory solid tumors. Three of patients on this study had renal cell carcinoma. While clinical responses were disappointing, treatment induced undetectable activity of DNMTs in renal cell carcinoma patients at low doses ($15mg/m^2$) [80]. An ongoing phase I/II clinical trial is assessing the feasibility, safety, and efficacy of anti-PD-1 antibody in combination with low-dose decitabine in patients with relapsed or refractory malignancies including RCC (NCT02961101). Another phase I/II trial of azacitidine in combination with bevacizumab for advanced RCC previously treated with VEGF- or mTOR-targeted therapy with or without prior immunotherapy was recently concluded and the results are pending (NCT00934440).

6.4 Prostate Cancer

Among all solid tumors evaluated in TCGA, prostate cancer has the lowest level of expression of *DNMT3A/B* (Figure 2). *GSTP1* is, however, frequently methylated gene in prostate cancer [95]. Functional epigenetic analyses in prostate cancer cell lines showed the reexpression of genes regulated by hypermethylation their promoters after treatment with zebularine or decitabine (*IFI6, GSTP1)* [95-97]. While zebularine failed to achieve the reexpression of *GCTP1* it effectively

reexpressed two GST detoxifying enzimes (GST-pi and GST-mu) in mice [96, 97]. Another commonly hypermethylated promoter in *ASC* gene (apoptosis associated speck-like protein) was demethylated and reexpressed by decitabine and zebularine in five prostate cancer cell lines [98]. Gertych *et al.* demonstrated that treatment with azacitidine and zebularine changed the DNA topology status in terms of DNA-histone complex decondensation, along with demethylating effects in prostate and liver cancer cells [99]. A study by Kim *et al.* described that overexpression of lysine-specific demethylase 4A (JMJD2A) resulted in initiation of prostate cancer development in mice further mediated by JMJD2A/ETV1/YAP1 pathway [100]. In further preclinical studies, azacitidine, demonstrated synergistic effects with docetaxel and cisplatin in androgen receptor (AR)-positive 22RV1 and in AR-negative PC3 cells [101]. Decitabine also exhibited synergy with cisplatin and cyclophosphamide in non-prostate cancer cell lines, although the relationship to induced DNA demethylation was unclear [102]. Prostate cancer cell lines that were treated with decitabine showed partial demethylation of *TMS1/ASC* locus (a frequently hypermethylated gene in prostate cancer) and the subset of alleles remained unmethylated for over 3 months while others were remethylated within 1 week [103]. Azacitidine prevented *de novo* development of cancer in all of 14 mice used in experiments with the model of transgenic adenocarcinoma of the mouse prostate [104].

Azacitidine and sodium phenylbutyrate failed to induce the response in a phase I trial including 5 prostate cancer patients, perhaps owing to the undetectable DNMT activity before the treatment [80]. Another phase I trial evaluating azacitidine and valproic acid where 2 patients with advanced prostate cancer were included reported stable disease in 1 patient [81]. In a phase II trial, subcutaneous azacitidine did not resensitize tumors to androgen-deprivation therapy in 36 patients with progressive castration resistant prostate cancer (CRPC) [105]. However, the combination of

azacitidine with docetaxel was active in metastatic CRPC patients [106]. In a phase I/II study, azacitidine was given daily for 5 days followed by docetaxel on day 6. PSA response was seen in 10 of 19 evaluable patients and objective response was observed in 3 of 10 evaluable patients. Significant demethylation of GADD45A was observed with azacitidine treatment [106].

Thus far, the clinical efficacy outcomes of azacitidine in prostate cancer trials have demonstrated only modest activity. Possible reasons may be the low level of DNTM3 expression or instability of DNA methylation inhibitors due to the short half life [107]. Wong *et al.* provided strong evidence for DNA methylation recovery and found that H3K9 trimethylation and H3K27 trimethylation were closely associated with DNA methylation recovery [108]. Overall, the efficacy of DNMTi in prostate cancer treatment is yet to be determined [2].

6.5 Penile cancer

Several studies summarized by Kuasne et al. attempted to describe epigenetic alterations in penile carcinoma (PeCa). Hypermethylation of the CDK2A gene promoter was found to be present in 15 to 42% of PeCa samples [109]. Feber et al. evaluated the methylation profile of 38 PeCa samples using high-density genome-wide methylation arrays. The authors identified a clear hypermethylation profile associated with cancer phenotype and identified novel epigenetic signatures associated with HPV infection and loco-regional spread. Interestingly, epigenetic signatures that were predictive of metastases in lymph nodes suggested a lower metastatic potential of hypermethylated tumors. In addition, HPV infection showed significant correlation with DNA methylation. The majority of HPV-positive samples were hypomethylated and showed better clinical outcome [110]. Distinct methylome and transcriptome patterns were described in more recent genome-wide methylation and transcriptome analysis. Aberrant DNA methylation was linked to

expression of specific genes connected to higher tumor aggressiveness and shorter duration of survival [111]. These studies suggest a role for DNA methylation in PeCa. Nevertheless, to our knowledge pre-clinical and clinical trials with DNMTis have not been reported.

Conclusion

Epigenetic targeting is an exciting new field in cancer research. Pre-clinical efforts to elucidate underlying mechanisms of treatment resistance have led to the initiation of several clinical trials involving epigenetic modulation in genitourinary malignancies. Current knowledge does not yet robustly support the immediate incorporation if DNMTis into treatment of genitourinary cancer. However, DNA methylation appears to be an important mechanism of treatment resistance, which may be overcome by incorporating DNMTis into the therapy. Initial studies evaluating older generations of DNMTis have shown insufficient activity due to unfavorable pharmacokinetics. Nevertheless, the discovery of new generation of DNMTis, coupled to a better understanding of mechanisms of action, have provided the rational for combination therapies, which may lead to more favorable clinical outcomes. Large clinical studies are needed to provide better understanding whether these agents will find place in everyday treatment of genitourinary cancer.

Compliance with ethical standards

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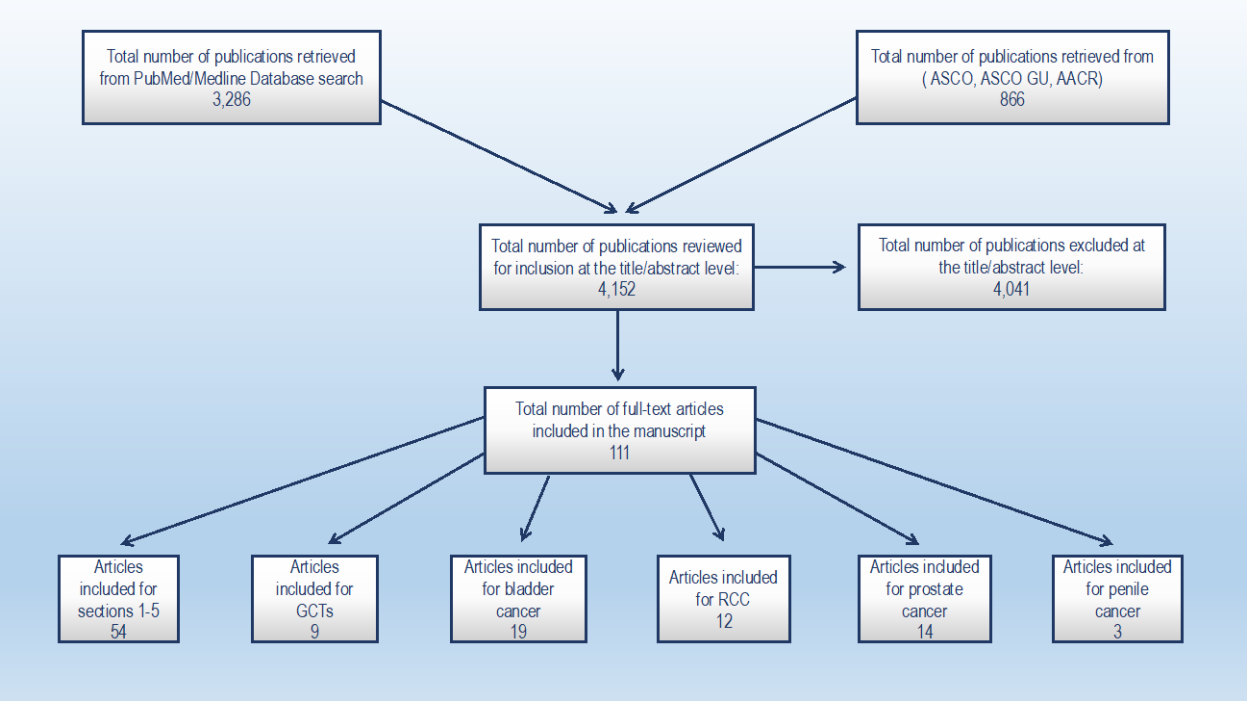
Figure 1. Schematic overview of the literature search.



Key words: epigenetics, DNMT, DNMTi, genitourinary, testicular cancer, germ-cell tumors, bladder cancer, renal cell carcinoma, prostate cancer, penile cancer, azacitidine, decitabine, guadecitabine, zebularine, non-nucleoside

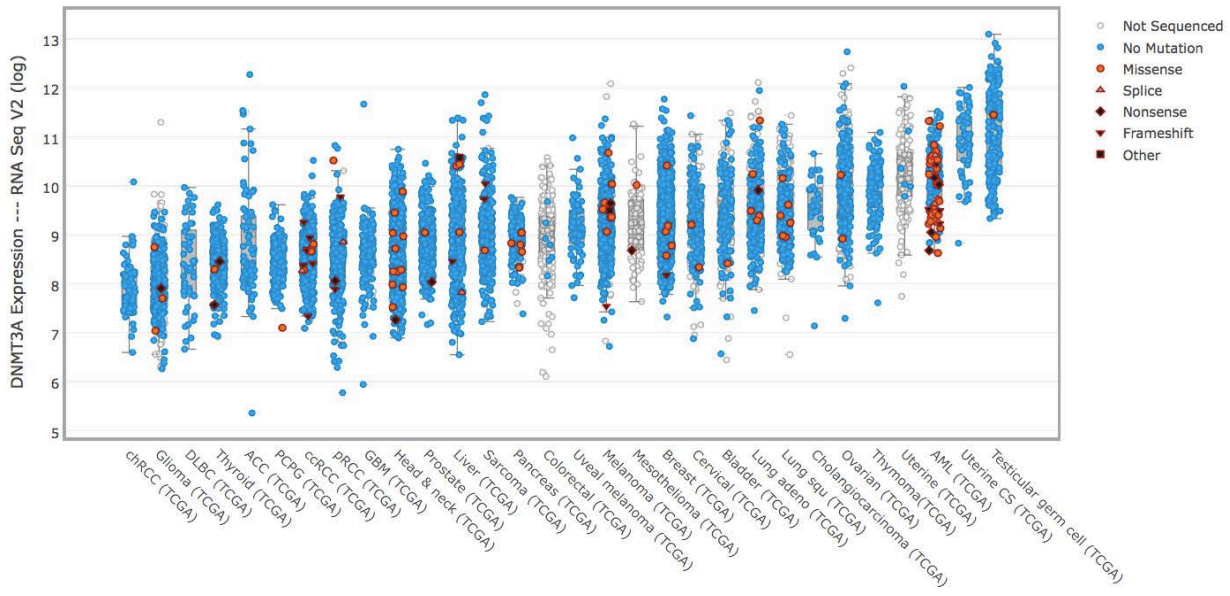
Abbreviations: ASCO – American Society of Clinical Oncology, ASCO GU – Genitourinary Cancers Symposium, AACR – American Association for Cancer Research, GCTs – germ cell tumors, RCC – renal cell carcinoma

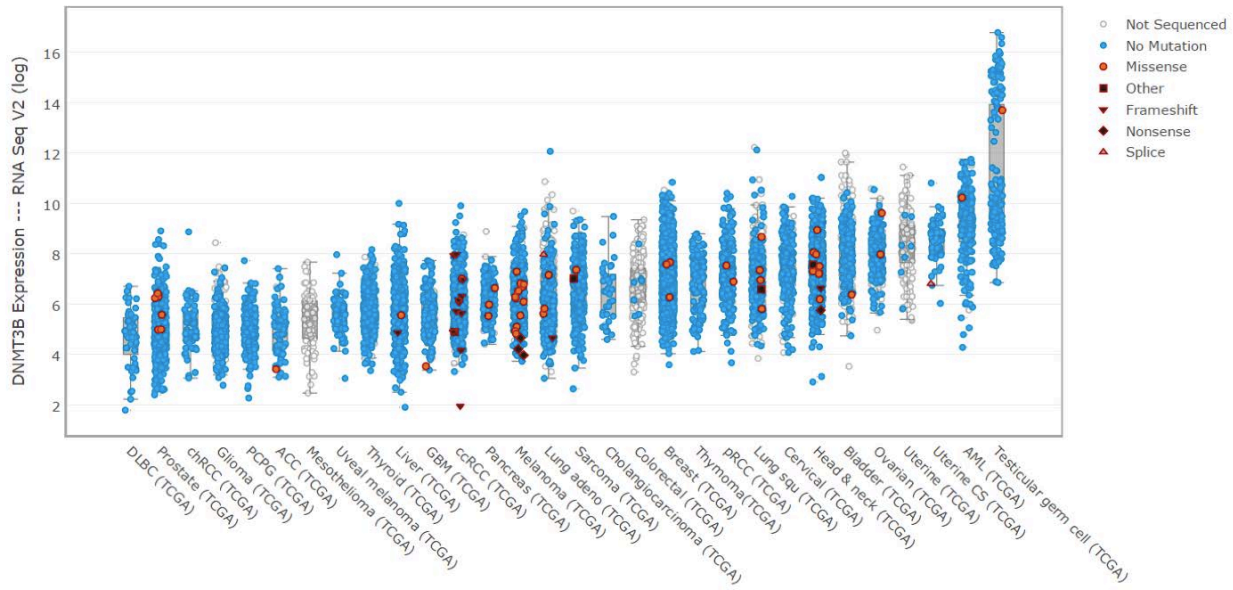
Table 1. Inclusion, exclusion criteria and the selection process for including publications inclusion in the review article

Inclusion criteria	*Exclusion criteria*
1. Pre-clinical *in vitro* studies evaluating DNA methyltransferase inhibitors (DNMTis) in genitourinary (GU) tumors including germ-cell tumors, bladder cancer, renal cell carcinoma, prostate cancer and penile cancer. 2. Pre-clinical *in vivo* studies evaluating DNMTis in GU tumors including germ-cell tumors, bladder cancer, renal cell carcinoma, prostate cancer and penile cancer.	1. Title including diagnoses, cell lines and animal models for cancers different than GU (e.g. hematologic malignancies, other solid tumors). 2. Abstract including cell lines, animal models and clinical trials for cancers different than GU. 3. Full text articles including cell lines, animal models and clinical trials for cancers other than GU tumors.

3. Clinical studies evaluating DNMTis in GU tumors including germ-cell tumors, bladder cancer, renal cell carcinoma, prostate cancer and penile cancer.	
Selection process of published works was conducted in several steps. First, review of publication titles retrieved from the literature search was performed. If title met the inclusion criteria, the abstract was reviewed. If abstract met the inclusion criteria, the full text article was reviewed. If inclusion criteria for full text article were met, the article was included in our review manuscript. If title of general nature was found (e.g. DNMTis in cancer, solid tumors etc.), the abstract was reviewed. Subsequently, if abstract did not clearly specify the cancer types, the full text article was reviewed and included or excluded according to the inclusion/exclusion criteria.	

Figure 2. The expression of DNMT3A/B in different types of tumors [58, 59]





Abbreviations: DLBCL – diffuse large B-cell lymphoma, chRCC – chromophobe renal cell cancer, PCPG – pheochromocytoma and paraganglioam, ACC – adrenocortical carcinomas, ccRCC – clear cell renal cell carcinoma, pRCC – papillary renal cell carcinoma, AML – acute myeloid leukemia, GBM - glioblastoma

Table 2. Ongoing clinical studies with DNMTis listed on *clinicaltrials.gov*

Study ID	*Phase*	*Treatments*	*Indication*	*Planned patients (N)*	*Date started*	*Planned completion date*
NCT02429466	I	SGI-110 Plus Cisplatin	Relapsed Platinum Refractory Germ Cell Tumors	15	April,2015	May 2018
NCT02223052	I	Oral Azacitidine	Adult Cancer Subjects including genitourinary cancers	60	October,2017	January 2018
NCT02788201	I/II	75 Approved Agents	Advanced Urothelial Carcinoma	20	May 20, 2016	June 29, 2019
NCT02961101	I/II	Anti-PD-1 antibody plus Decitabine	Relapsed or Refractory Malignancies Including RCC	100	May 2016	May 2020

NCT01799083	I/II	Decitabine Alone and/or in Combination with Chemotherapy and/or Cytokine Induced Killer Cell Transfusion	Relapsed or Refractory Solid Tumors and B Cell Lymphomas	100	December 2012	December 2017
NCT02423057	I	4'-Thio-2'-Deoxycytidine	Advanced Solid Tumors	46	April 13, 2015	April 18, 2018
NCT00978250	II	5-Fluoro-2'-Deoxycytidine with Tetrahydrouridine	Solid tumors including urinary bladder neoplasms	165	August 20, 2009	May 27, 2017
NCT02959437	I/II	Azacitidine plus Pembrolizumab and Epacadostat	Advanced Solid Tumors	142	January 24, 2017	October 2021
NCT02998567	I	Guadecitabine plus Pembrolizumab	Castration-Resistant Prostatic Cancer and Non-Small Cell Lung Cancer	35	December 2016	November 2018
NCT02030067	I/II	Single Agent RX-3117	Metastatic Bladder and Pancreatic Cancer	72	December 2013	December 2017