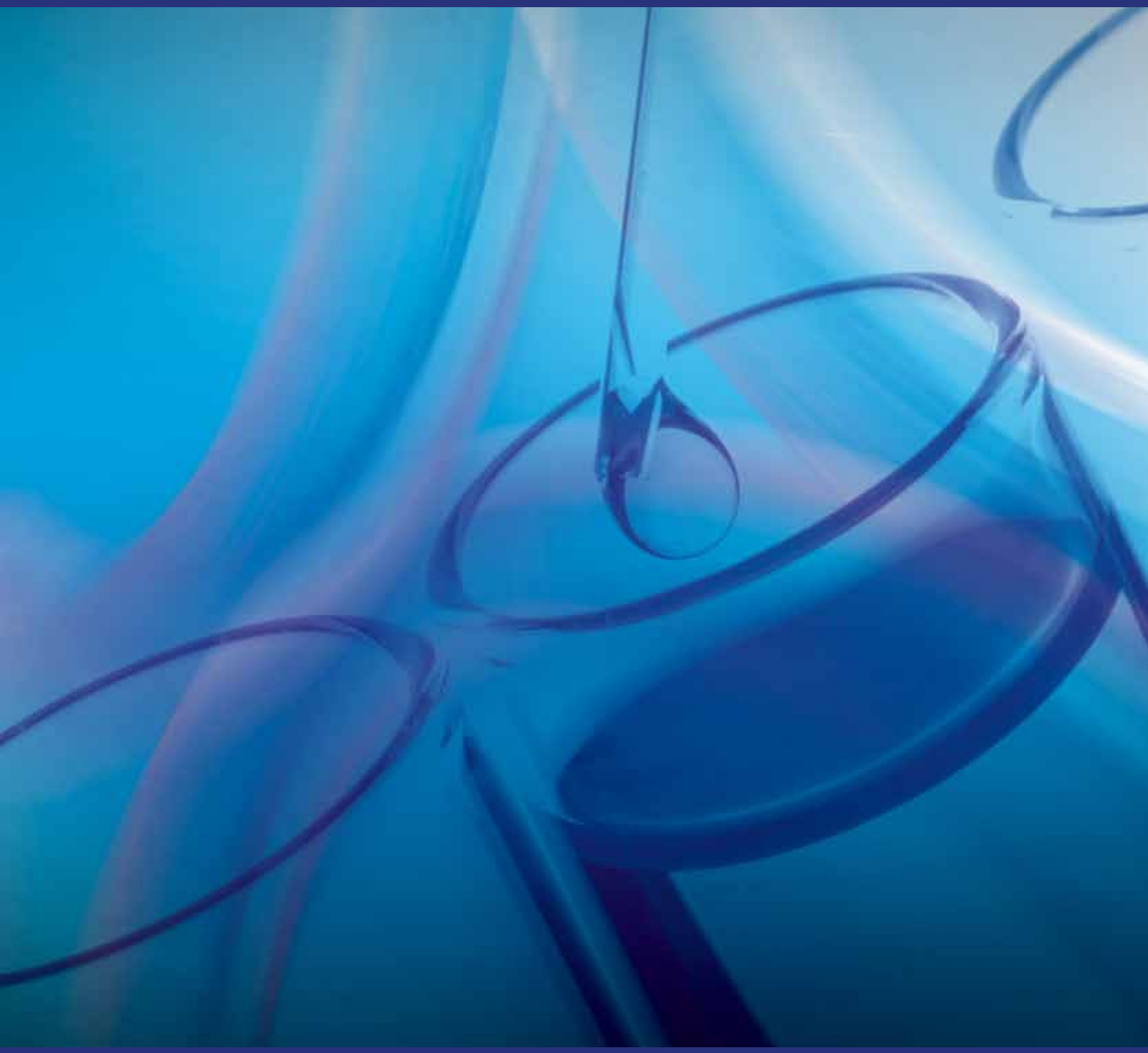
Advances in Urology

Urothelial Carcinoma

Guest Editors: Nan-Haw Chow, Margaret Knowles, and Trinity J. Bivalacqua



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Editorial Board

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Editorial
Urothelial Carcinoma

Nan-Haw Chow,[1] Margaret Knowles,[2] and Trinity J. Bivalacqua[3]

[1] *Department of Pathology, College of Medicine, National Cheng Kung University, No.1 University Road, Tainan City 701, Taiwan*
[2] *Leeds Institute for Molecular Medicine, Section of Experimental Oncology, Cancer Research UK Clinical Centre, St Jame's University Hospital, Beckett Street, Leeds LS9 7TF, UK*
[3] *Johns Hopkins Medical Institutions, The James Buchanan Brady Urological Institute, 600 N. Wolfe Street, Marburg 409, Baltimore, MD 21287, USA*

Correspondence should be addressed to Nan-Haw Chow, chownh@mail.ncku.edu.tw

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Bladder cancer is a common urologic cancer. In North America, South America, Europe, and Asia, the most common type of epithelial tumor diagnosed is urothelial (transitional cell) carcinoma. Worldwide, however, squamous cell carcinoma is the most common form of bladder cancer, accounting for 75% of cases in developing nations. Clinical management of this type of bladder cancer is challenging due to the heterogeneity in bladder tumors with respect to invasion and metastasis and frequent recurrence in the bladder among patients treated with bladder preservation therapies. These dilemmas of clinical practice have stimulated translational research. At the same time, the characteristics of environment-driven carcinogenesis and divergent molecular pathways in the development of low- and high-grade tumors provide a unique opportunity for molecular research in cancer biology. Bladder cancer is also at the forefront of biomarker development because of the ease of developing noninvasive urine tests.

This special issue presents the up-to-date summaries related to molecular pathogenesis, diagnosis, prognostic assessment, and management of bladder cancer. The editorial board selected 8 papers that address these special research areas in the urothelial carcinoma. In the basic research section, Han et al. describe the methylation pattern of noninvasive and invasive bladder cancers, respectively. It appears that two epigenetic pathways give rise to two tumor types, and certain epigenetic alterations precede histopathological changes. The dynamic nature and reversibility with pharmacological interventions make it an excellent target for epigenetic therapy in the future.

Martino et al. provide a comprehensive review of the molecular alterations of fibroblast growth factors receptors in urothelial carcinogenesis. They underscore the potential of the receptors and downstream signaling pathways as therapeutic targets, diagnostic and prognostic markers, and screening tools for early detection and clinical management of urothelial cancer.

Another novel treatment for urothelial carcinoma is described by K. G. Potts et al. They review recent advancements of therapy using oncolytic viruses engineered to selectively replicate in and lyse tumor cells leaving normal cells unharmed. Although encouraging safety profiles and antitumor activity have been demonstrated with a variety of oncolytic viruses, the ultimate proof still needs to be provided by randomized Phase III clinical trials.

With respect to molecular diagnosis of bladder cancer, Reinert et al. discuss the currently available biomarkers NMP22, ImmunoCyt, and UroVysion. All of three markers have a higher sensitivity than cytology, but a lower specificity. This review focuses on the urinary DNA methylation markers in the diagnosis and surveillance of bladder cancer.

With respect to medical treatment, Askeland et al. summarize the updated progress of immunotherapy for nonmuscle invasive bladder cancer. The agents include INF-α, IL-2, IL-12 and IL-10, either as adjuncts with *Mycobacterium bovis* bacillus Calmette-Guérin treatment or as a solo replacement therapy.

Tsai et al. report a meta-analysis to examine the evidence related to the predictive importance of ErbB receptor signaling in bladder cancer. They demonstrate that the overall

risks of disease progression for patients with EGFR or ErbB2 overexpression are 4.5 and 1.1, and the risks of mortality are 3.0 and 1.1, respectively. Future direction of marker assessment should focus on the significance of coexpression patterns of the ErbB receptor family.

Mohamed et al. discuss the impact of different treatment options for muscle invasive bladder cancer patients on the quality of life in the short and long term, particularly concerning urinary diversion. They also call attention to the challenges that patients and family caregivers may face, including psychological, physical and social daily living, emotional support, and interpersonal communication.

A paper contributed by Hyndman et al. presents the novel technology for neo-urinary conduit seeded with autologous smooth muscle cells. This construct may potentially eliminate the complications associated with the use of gastrointestinal segments in urinary reconstruction and greatly facilitate recovery from cystectomy.

It is hoped that this special issue can bring the latest information to all urologists, pathologists, and medical oncologists who wish to provide their patients with the most comprehensive care.

Nan-Haw Chow
Margaret Knowles
Trinity J. Bivalacqua

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Review Article

Clinical Significance of ErbB Receptor Family in Urothelial Carcinoma of the Bladder: A Systematic Review and Meta-Analysis

Yuh-Shyan Tsai,[1] Hong-Lin Cheng,[1] Tzong-Shin Tzai,[1,2] and Nan-Haw Chow[3]

[1] *Department of Urology, National Cheng Kung University Hospital, Tainan 70403, Taiwan*
[2] *Department of Urology, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan*
[3] *Department of Pathology, College of Medicine, National Cheng Kung University Hospital, Tainan 70403, Taiwan*

Correspondence should be addressed to Tzong-Shin Tzai, tts777@mail.ncku.edu.tw and Nan-Haw Chow, chownh@mail.ncku.edu.tw

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The prognostic importance of examining ErbB receptor family expression in human bladder cancer remains uncertain. Using published evidence, we examined the clinical value and the updated results of clinical trials targeting ErbB receptor family members. Twenty-seven articles from 65 references related to ErbB receptor expression assessment in bladder cancer were reviewed. The estimates included the association significance, hazard ratios, and 95% confidence intervals (CIs) from actuarial curves and survival analyses. A meta-analysis was done on those reports using univariate log-rank tests or a Cox-regression model. The methods of analysis and study subjects chosen varied widely among studies. The overall risks of disease progression for patients with EGFR or ErbB2 overexpression were 4.5 (95% CI: 2.5–8.4) and 1.1 (95% CI: 0.6–1.9), and the risks of mortality were 3.0 (95% CI: 1.6–5.9) and 1.1 (95% CI: 1.0–1.2), respectively. However, the significance of coexpression patterns of the ErbB receptor family remains controversial. None of six clinical trials yielded convincing results for blockading ErbB receptor signaling in urothelial carcinoma. The results of this analysis suggest that assessing co-expression patterns of the ErbB family may provide better prognostic information for bladder cancer patients.

1. Introduction

One characteristic of bladder cancer is its variable patient prognosis. About 70% of superficial (Ta and T1) tumors recur, and 10–20% of them become invasive [1]. Tumors that are invasive at primary diagnosis carry a high risk of progression despite radical cystectomy and other auxiliary treatments. Conventional prognostic factors, such as tumor stage, grade, size, and multifocality, do not accurately predict the clinical outcome for some patients. Therefore, extensive efforts have been made to identify biomarkers for predicting disease progression, response to treatment, and chance of long-term survival. Currently, it is recommended that patients with bladder cancer have regular urinary cytology, cystoscopy, and imaging studies at followup [2].

The ErbB receptor family (also known as the epidermal growth factor receptor (EGFR) family) is a major class of receptor tyrosine kinase (RTK) protooncogenes. They are important in many cell regulatory processes, such as proliferation, migration, adhesion, and, potentially, cellular transformation, including urothelial carcinogenesis. The ErbB family consists of 4 members: ErbB1 (also called EGFR and HER1), ErbB2 (c-*erb*B-2 and HER2/*neu*), ErbB3 (HER3), and ErbB4 (HER4). Dimerization by binding two monomers is the regulatory mechanism for activating RTKs [3]. In some cases, the formation of heterodimeric complexes allows interaction and crosstalk between different receptors of the same subfamily, and the ErbB receptor family is the best example of homo- and heterodimerization *in vivo* [4, 5]. Therefore, clarifying the clinical significance of ErbB

expression may provide important molecular targets for cancer therapy.

EGFR signaling regulates biological processes important for the pathogenesis of human cancers, including lung cancer, breast cancer, and prostate cancer [6]. In practice, therapy that targets EGFR gene mutations in primary tumors has extended the theme of targeted cancer therapies [7]. In breast cancer, HER2 amplification status is a pivotal biomarker in predicting response to chemotherapy [8], and a humanized anti-HER2 monoclonal antibody (trastuzumab) improved the survival of HER2-positive breast cancer patients [9]. The prognostic significance of ErbB receptor signaling has tissue-specific relevance. For example, EGFR/HER2-MAPK axis is important in human breast cancer while the kinase activity of the HER2/ErbB3 axis plays a major role in the DNA binding and androgen receptor stability in prostate cancer [10]. Moreover, the EGFR inhibitor gefitinib is ineffective in treating hormone-refractory prostate cancer, a result questioning the significance of the EGFR/HER2 axis in the molecular pathogenesis of prostate cancer [11].

To establish the clinical relevance of ErbB receptor family members in bladder cancer, we systematically reviewed the papers published in the past two decades on ErbB receptor family expression, either one of the members or the coexpression patterns, and their impact on patient prognosis. Our objectives were to confirm the significance of ErbB receptor expression in predicting recurrence, progression, and mortality in patients with bladder cancer, to identify factors that might affect the prognostic evaluation of ErbB receptors, and to conduct a meta-analysis of available estimates. In addition, we assessed the potential sources of heterogeneity underlying the conflicting results, and incorporated quantitative methods to analyze the data. The updated results of clinical trials targeting ErbB receptor signaling were also reviewed.

2. Materials and Methods

2.1. Search Strategy and Selection Criteria. Original articles published between January 1985 and May 2011 showing prognostic significance of expression or amplification of ErbB receptor members in patients with bladder cancer were systematically reviewed. Using the keywords "EGF", "EGFR," "c-erb-B1," "c-erb-B2," "c-erb-B3," "c-erb-B4," "neu," "epidermal growth factor," and "bladder neoplasms or transitional cell carcinomas-in-humans," we identified 710 relevant articles in the PubMed database. The number of studies was reduced to 65 by limiting the search to "prognosis" or "survival," including disease recurrence and progression. Duplicate data, identified in the same cohort by reviewing the interstudy similarities of investigators, source of patients, recruitment period, or inclusion criteria, were excluded from the current analysis. Only the largest series were included in our analysis.

2.2. Data Extraction, Handling, and Analyses. Our database was designed to ensure the breadth of relevant data obtained, based on study design, patient outcomes, tumor characteristics, statistical analyses, biological samples, analytical methods (namely, immunohistochemistry [IHC], fluorescence in situ hybridization [FISH], and real-time polymerase chain reaction [RT-PCR]), and the incidence of overexpression or gene amplification of ErbB receptor family members.

Two methods were used to summarize the results. Because most articles provided only the P values or statements of whether results were significant (and no other measures of effect), the analyses of recurrence, progression, mortality, progression-free survival, disease-free survival, and resistance to chemotherapy and radiotherapy were based on definitions in the original reports. Briefly, recurrence was defined as a tumor that reappeared in the urinary bladder not at a higher T stage, while disease progression was defined as any tumor with a higher T stage in the local tumor, node, or metastasis. The P values (or statements of significance) were extracted from association analyses (χ^2 test, Fisher's exact test, Student's t-test, the Mann-Whitney U-test, and logistic regression), and the risk estimates and 95% confidence intervals (CIs) from univariate (Kaplan-Meier curves and the log-rank test) and multivariate survival (Cox regression) analyses. Data are summarized by providing the means of percentages or actual numbers with either the standard deviation (SD) or range. χ^2 tests and Fisher's exact tests were used, when appropriate, to assess the independence of two categorical variables. Unconditional logistic regression models were used to identify the significance of study characteristics ($P < 0.05$).

A meta-analysis using multivariate tests was then done. Wolf's method was used to combine the risk estimates by applying the inverse of variance as the weighting factor. Potential sources of heterogeneity were investigated using graphical methods, such as the Galbraith plot. A heterogeneity test based on the statistics was done in all meta-analyses. The heterogeneity was considered significant when $P < 0.10$. In cases of substantial heterogeneity, random-effect models were used. The extent to which the combined risk estimate was affected by individual study was examined by consecutively omitting every study from the meta-analysis. Metaregression was used to explain the potential heterogeneity from the same characteristics included in the P value analysis. The publication bias was investigated using Egger's and Begg's graphical methods. The analyses were done using Comprehensive Meta Analysis Version 2 (Biostat, Englewood, NJ, USA). Significance was set at $P < 0.05$ (two sided).

3. Results

3.1. Significance of Individual ErbB Receptor Expression. Tumors recurred in 1233 patients in 16 analyses reported in 8 studies (median: 162 patients; range: 52–243) (Table 1) [5, 12–18]. The receptor members analyzed in order of frequency were ErbB2 (7 of 8) and EGFR (6 of 8). Three studies [5, 15, 16] reported significantly positive results. After data processing, only 4 reports were accepted for analysis: 2 for EGFR and 2 for ErbB2 (Figure 1(a)). The distribution of the tumor stages in patients with cancer varied widely. All four studies used IHC to analyze their study samples. Overall, the hazard ratios of tumor recurrence were 1.588 (95% CI: 0.9–2.7).

Table 1: Significance of ErbB receptor family as a marker for tumor recurrence.

Study name	Methods	Pts no.	Study subject	Significance
EGFR				
[12]	IHC	230	T0-1	NS
[13]	IHC	73	T2–4	NS
[14]	IHC	141	T0-1	NS
[15]	IHC	52	T0-1	Yes
[16]	IHC	182	T0–4	Yes
[5]	IHC	245	T0–4	NS
ErbB2				
[16]	IHC	182	T0–4	NS
[12]	IHC	230	T0-1	NS
[13]	IHC	55	T2–4	NS
[14]	IHC	141	T0-1	NS
[17]	IHC	62	T0-1	NS
[18]	IHC	248	T0-1	NS
[5]	IHC	245	T0–4	Yes
ErB3				
[16]	IHC	128	T0–4	NS
[5]	IHC	245	T0–4	Yes
ErbB4				
[16]	IHC	124	T0–4	NS

IHC: immunohistochemistry.

For cancer progression, 484 patients from 9 analyses were reported in 8 studies (median: 60 patients; range: 21–113) (Table 2) [15, 19–24]. The receptor members analyzed in order of frequency were ErbB2 (5 of 8) and EGFR (4 of 8). Four studies [15, 19, 20, 22] found significantly positive results. After data processing, only 4 reports on EGFR and 3 on ErbB2 were accepted for analysis (Figure 1(b)). The distribution of tumor staging varied widely. All four studies used IHC to analyze their study samples. Overall, the hazard ratios of cancer progression were 4.611 (95% CI: 2.5–8.4) for EGFR and 1.067 (95% CI: 0.59–1.97) and ErbB2 overexpression.

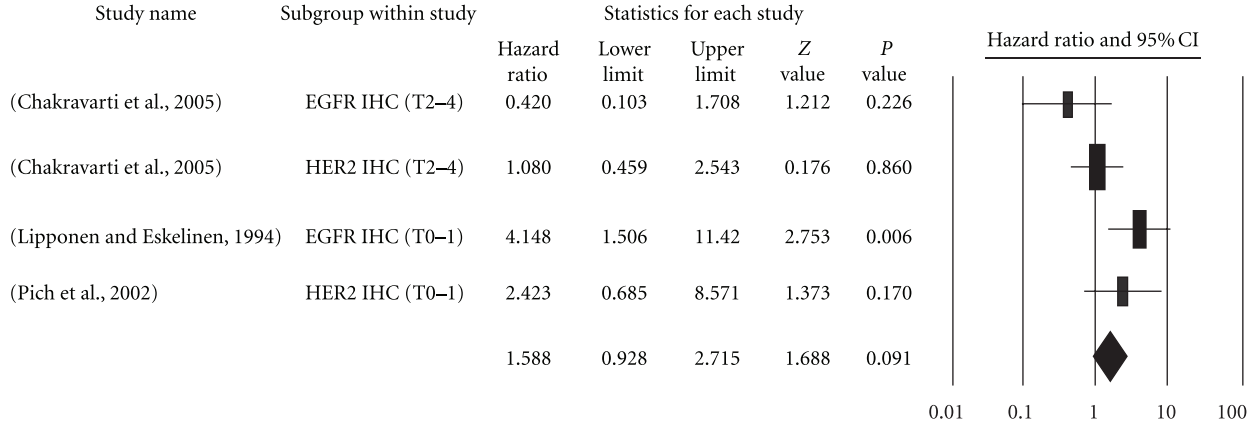
Cancer-related mortality in 3444 patients from 31 analyses was reported in 20 studies (median: 88 patients, range: 39–1500) (Table 3) [5, 13, 15, 16, 19–27, 34–37]. The receptor members analyzed in order of frequency were ErbB2 (18 of 20) and EGFR 8 of 20). Thirteen studies [5, 13, 15, 16, 21–23, 26, 34–37] reported significantly positive results. After data processing, only 3 reports on EGFR and 10 on ErbB2 were accepted for analysis (Figure 2). The distribution of tumor staging also varied widely. IHC was the most common method of assessment; however, FISH was used in 1 study, and gene copy amplification was used in another. Overall, the hazard ratios of cancer death were 3.044 (95% CI: 1.6–5.9) for EGFR and 1.090 (95% CI: 1.0–1.2) for ErbB2 overexpression.

3.2. Significance of the Coexpression Pattern of ErbB Receptors. Three studies [5, 16, 28] investigated the significance of the coexpression patterns of ErbB receptor family members in association with tumor recurrence and patient survival. None of the coexpression patterns were significant in predicting tumor recurrence. In contrast, several coexpression patterns were independent prognostic indicators of bladder cancer death, namely, EGFR-ErbB2, ErbB2-ErbB3 [5], and high EGFR or ErbB2 plus low ErbB3 or ErbB4 [16, 28] (Table 4).

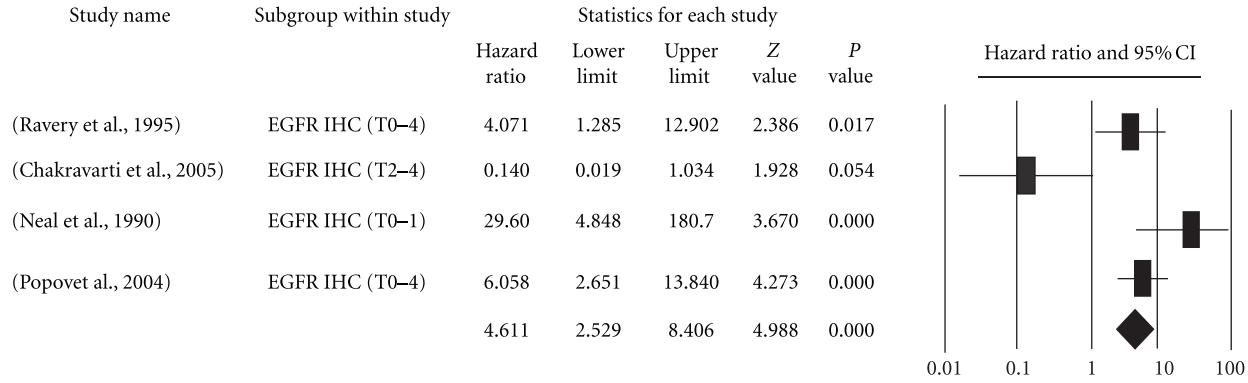
3.3. Assessment and Publication Bias. A substantial funnel plot asymmetry suggestive of the publication bias was revealed for ErbB2 (Figure 3(b)). However, no obvious funnel plot asymmetry was found for EGFR, possibly because only 4 studies were analyzed (Figure 3(a)).

3.4. Clinical Trials of Targeting ErbB Signaling in Human Bladder Cancer. Until February 2012, there were 6 finished clinical trials targeting ErbB signaling in human bladder cancer (Table 5). Cetuximab is a chimeric (mouse/human) monoclonal antibody for blocking EGFR signaling. A phase II clinical trial [38] of 39 pretreated patients with metastatic urothelial carcinoma demonstrated that combined cetuximab and paclitaxel chemotherapy yielded a better response rate (28.5%) than did cetuximab monotherapy, in which 9 of 11 patients had disease progression at 8 weeks.

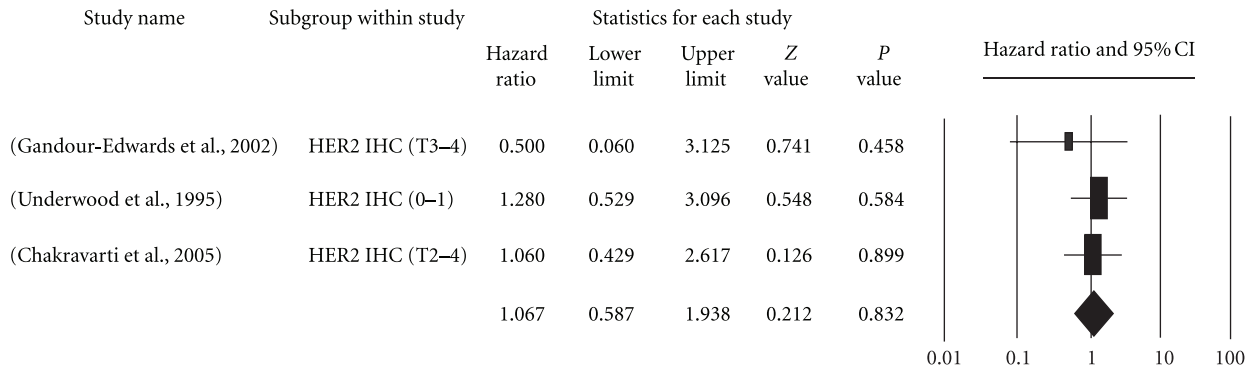
Gefitinib is the first selective inhibitor of the EGFR tyrosine kinase domain. An overall response rate of 42.6% (95% CI: 29.2–56.8%) was demonstrated in a phase II trial of 58 patients with metastatic urothelial carcinoma treated with gemcitabine and cisplatin chemotherapy plus gefitinib [39, 40], the median survival time was 15.1 months (95% CI: 11.1–21.7 mo), and time to progression was 7.4 months (95% CI: 5.6–9.2 mo). Twenty-five patients completed the



(a) Bladder cancer recurrence



(A) EGFR



(B) ErbB2

(b) Bladder cancer progression (EGFR)

Figure 1: Forest plots of studies on the prognostic significance of EGFR and ErbB2 overexpression in bladder cancer. (a) The effect on recurrence. (b) The effect of EGFR (A) or ErbB2 (B) on progression. Hazard ratios and 95% (confidence intervals) CIs for patients with either EGFR- or ErbB2-positive tumors.

trial without reducing or discontinuing the gefitinib. The authors concluded that this combination therapy was well tolerated and effective in metastatic disease. However, adding gefitinib did not improve the response rate or patient survival. In contrast, a phase II trial of 31 pretreated patients with metastatic urothelial carcinoma [41] reported a response rate of 6.5%, and the authors concluded that gefitinib is ineffective as a second-line agent for metastatic urothelial carcinoma.

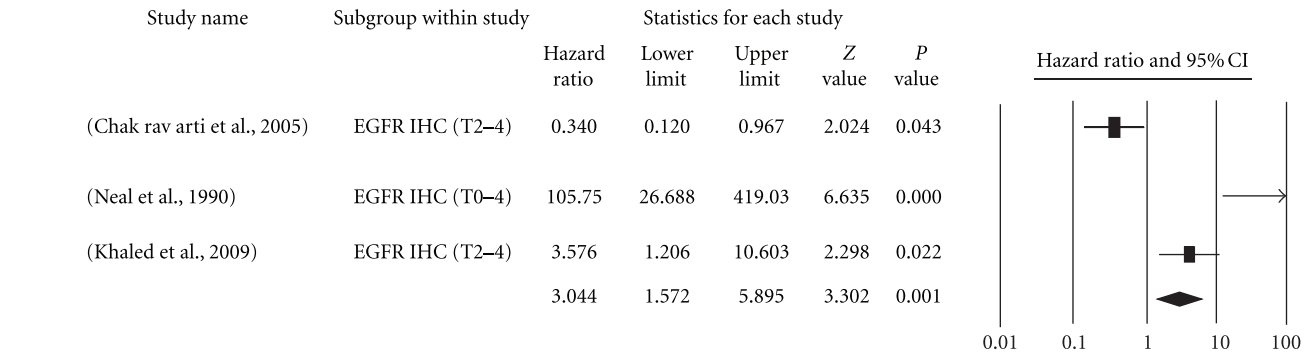
Lapatinib is an EGFR and ErbB2 dual tyrosine kinase inhibitor. An objective response rate (ORR) greater than 10% was found in only 1 of 59 patients (1.7%) in a phase II trial using lapatinib as the second-line agent for patients with metastatic urothelial carcinoma [42], and the disease

Table 2: Significance of ErbB receptor family as a marker for cancer progression.

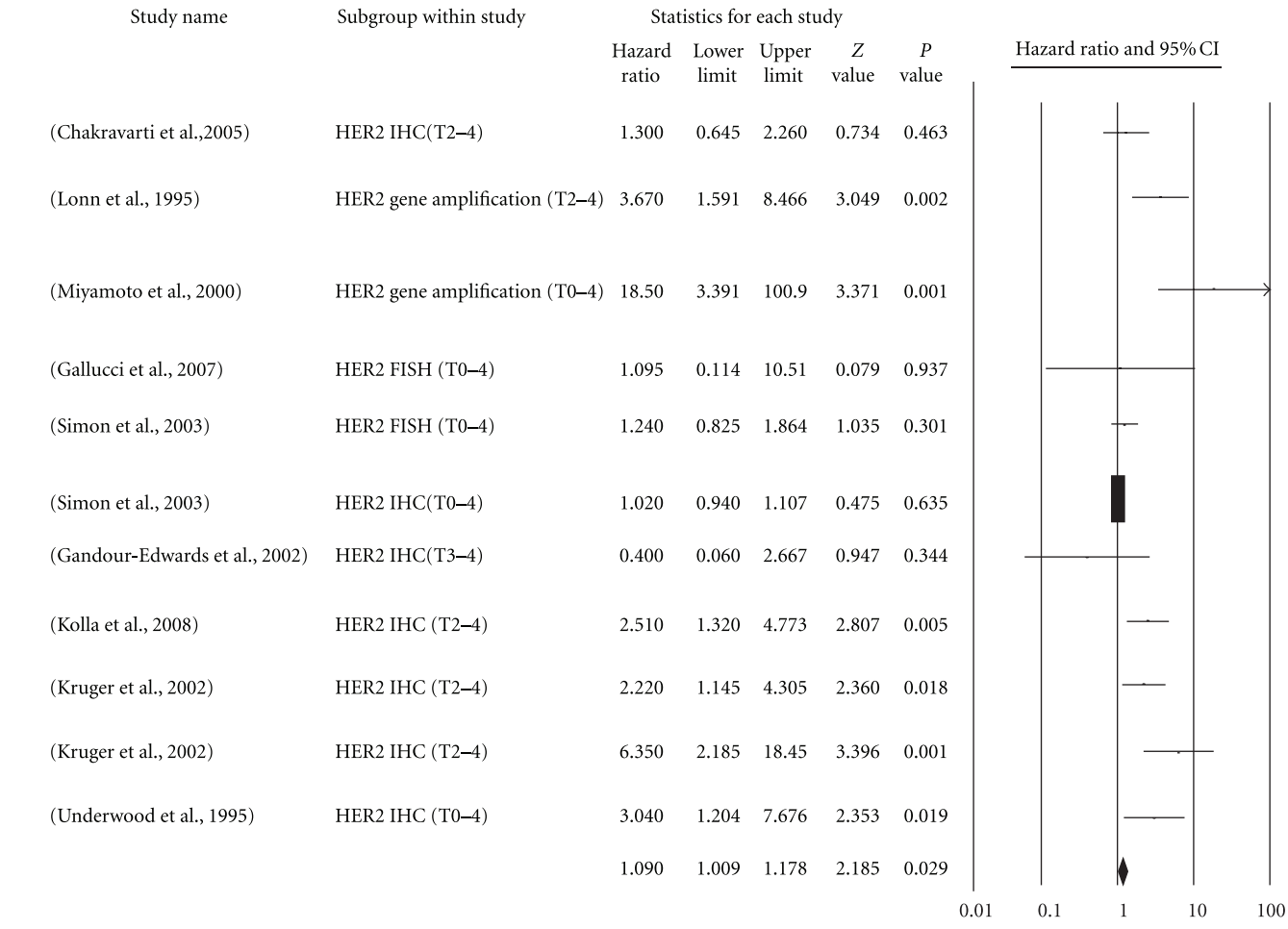
Study name	Methods	Pts no.	Study subject	Significance
EGFR				
[19]	IHC	57	T0–4	Yes
[13]	IHC	73	T2–4	NS
[15]	IHC	52	T0-1	Yes
[20]	IHC	113	T0–4	Yes
ErbB2				
[21]	FISH	62	T0–4	NS
[13]	IHC	55	T2–4	NS
[22]	IHC	39	T3-4	Yes
[23]	IHC	21	T0-1	NS
[24]	IHC	67	T2–4	NS

Table 3: Significance of ErbB receptor family as a marker for bladder cancer death.

Study name	Methods	Pts no.	Study subject	Significance
EGFR				
[13]	IHC	73	T2–4	Yes
[25]	IHC	109	T2	NS
[16]	IHC	182	T0–4	NS
[26]	IHC	59	T2–4	Yes
[27]	IHC	141	T2–4	NS
[15]	IHC	52	T0-1	NS
	IHC	101	T0–4	Yes
[26]	RT-PCR	59	T2–4	Yes
[28]	RT-PCR	88	T0–4	NS
[29]	qRT-PCR	73	T0–4	NS
ErbB2				
[30]	Gene amplification	163	T0–4	Yes
[31]	Gene amplification	57	T0–4	Yes
[21]	FISH	62	T0–4	NS
[32]	FISH	1500	T0–4	NS
[13]	IHC	55	T2–4	NS
[22]	IHC	39	T3-4	Yes
[25]	IHC	109	T2	NS
[33]	IHC	80	T2–4	NS
[16]	IHC	184	T0–4	NS
[34]	IHC	90	T2–4	Yes
[35]	IHC	138	T2–4	Yes
[36]	IHC	132	T2–4	Yes
[27]	IHC	141	T2–4	NS
[37]	IHC	88	T0–4	Yes
[32]	IHC	1500	T0–4	NS
[5]	IHC	245	T0–4	Yes
[23]	IHC	89	T0–4	Yes
[28]	RT-PCR	88	T0–4	NS
[29]	RT-PCR	73	T0–4	NS
ErbB3 or ErbB4				
[16]	IHC	128	T0–4	NS
	IHC	124	T0–4	Yes



(a) EGFR for bladder cancer death



(b) ErbB2 for bladder cancer death

Figure 2: Forest plots of prognostic significance of EGFR and ErbB2 overexpression on bladder cancer death. (a) EGFR. (b) ErbB2. Hazard ratios and 95% (confidence intervals) CIs for patients with either EGFR- or ErbB2-positive tumors.

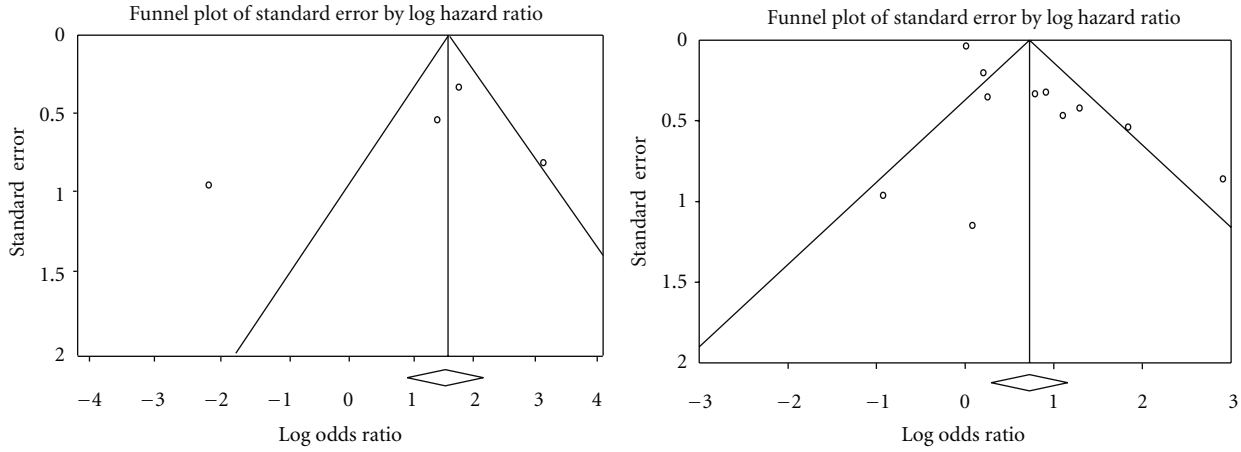
was stabilized in 18 of these patients (31%). The result is basically negative. Interestingly, the clinical advantage (ORR and stable disease) correlated with the EGFR overexpression ($P = 0.029$), and, to some extent, HER-2 overexpression.

Vandetanib is a tyrosine kinase inhibitor for EGFR, vascular endothelial growth factor receptor (VEGFR), and (rearranged during transfection) RET. Choueiri et al. reported that combined vandetanib and docetaxel did not provide more benefit for ORR or patient survival than did docetaxel plus placebo in a double-blind trial of 142 patients with metastatic urothelial carcinoma. The toxicity was also greater in the combination group [43]. Taken together, the outcome of current clinical trials suggests that more investigations are required to identify an appropriate strategy or a more effective agent targeting ErbB signaling in the design of treatment for patients with urothelial carcinoma.

Table 4: Co-expression of ErbB receptor family as a marker for bladder cancer prognosis.

Study name	Methods	Pts no.	Study subject	Significant co-expression pattern for	
				Recurrence	Survival
[5]	IHC	245	T0–4	EGFR-ErbB2-ErbB3*	EGFR-ErbB2 ErbB2-ErbB3
[16]	IHC	184	T0–4	Not significant	High EGFR + low ErbB4
[28]	RT-PCR	88	T0–4	Not done	High EGFR + low ErbB3 or ErbB4 High ErbB2 + low ErbB3 or ErbB4

*$P = 0.075$.



(a) EGFR for bladder cancer progression

(b) ErbB2 for bladder cancer death

Figure 3: Funnel plots of publication bias for EGFR and ErbB2. (a) No obvious funnel plot asymmetry was found for EGFR because only four studies were analyzed. (b) A substantial funnel plot asymmetry for ErbB2 was found, which suggests the existence of publication bias.

4. Discussion

This meta-analysis revealed that estimates of the significance of ErbB receptor family member expression vary substantially between studies. Nonetheless, EGFR overexpression is moderately predictive of progression and mortality in patients with bladder cancer. ErbB2 overexpression is weakly predictive of cancer mortality. Since relatively few studies have examined the implications of ErbB3 and ErbB4, no conclusion can be made at this stage. However, these findings should be interpreted carefully because relatively few studies were eligible for analysis.

It has been more than three decades since the discoveries of EGF and EGFR [44, 45]. Many bladder cancer studies examined the signaling events of this receptor family [16, 46, 47]. Given that human urine contains high concentrations of EGF (20 ng/mg creatinine) [48] and transforming growth factor (TGF)-α (0.6 ng/mg creatinine) [49], the interaction of ligands with their cognitive receptors has been thought crucial in the homeostasis of bladder mucosa. This hypothesis is supported by clinical observations that urinary EGF is inversely correlated with the intensity of EGFR expression in primary tumors ($P = 0.04$) [47]. The finding supports the importance of the urinary EGF and urothelial EGFR interaction in the pathogenesis of human bladder cancer.

When ligands bind to the extracellular domains of specific RTKs, tyrosine kinase activity at their intracellular domain is activated [50]. Several regulatory signaling pathways of the ErbB receptor family are important in the proliferation, angiogenesis, migration, and metastasis of human cancer [51]. The conventional paradigm is that aberrant activation of ErbB receptors is generated by the overexpression or mutations of the receptor, and by the autocrine production of ligands [50]. It is now known that signaling output from EGFR is quite complicated. Not only tyrosine-phosphorylated EGFR engages at least six biochemical downstream pathways, but EGFR family RTKs include three other receptors, of which only ErbB4 is autonomous [52]. Other ErbB proteins may be coexpressed and activated in the same cell, resulting in their dimerization with EGFR. The association of EGFR with ErbB2 prevents its downregulation, which reinforces the biological effects of EGFR [53].

ErbB signaling may be terminated or blunted by other mechanisms, such as the dissociation of ligands, which causes dephosphorylation or degradation of the receptors [54, 55]. Alwan et al. hypothesized that proteasomal targeting of ErbB proteins or lysosomal degradation upon ligand-induced endocytosis is involved in EGFR downregulation [56]. Since the ErbB receptor family has many negative feedback mechanisms (e.g., receptor endocytosis, phosphorylation, and ubiquitination/deubiquitination) and crosstalk with other pathways (such as NOTCH pathways, VEGF, and TGF-β), components of the EGFR/ErbB network are excellent targets for cancer therapy [57].

Table 5: The update results of published clinical trials targeting EGFR signaling in urothelial carcinoma patients.

Study agents	Pts no.	ORR (%)	Recommendation
EGFR signaling			
Cetuximab + paclitaxel versus cetuximab	39	28.5 versus 18	The combination merits further evaluation [38]
Geftinib + Gemcitabine, cisplatin	58	42.6%	The combination of cisplatin, gemcitabine, and gefitinib is well tolerated, and the addition of gefitinib does not appear to improve response rate or survival [39, 40]
Geftinib	31	6.5	Geftinib (ZD1839) is ineffective as a second-line agent for urothelial carcinoma [41]
EGFR and ErbB2 signaling			
Lapatinib	59	ORR (1.7%) and SD (31%)	A negative result, but clinical benefit (ORR and SD) is correlated with EGFR overexpression ($P = 0.029$), and, to some extent, HER-2 overexpression [42]
EGFR, VEGFR, and RET signaling			
Vandetanib plus docetaxel versus placebo plus docetaxel	142	ORR, 7 versus 11 ($P = 0.56$); PFS, 2.6 m versus 1.6 m ($P = 0.939$); OS, 5.9 m versus 7.0 m ($P = 0.347$)	The addition of vandetanib to docetaxel did not result in a significant improvement in PFS, ORR, or OS [43]

ORR: objective response rate; SD: stable disease, PFS: progression-free survival; OS: overall survival; VEGFR: vascular endothelial growth factor receptor; RET: rearranged during transfection.

Several studies have reported that overexpressed or mutant EGFR family members drive the development of human cancers, including lung [58], breast [59], melanoma [60], prostate [61], and urinary bladder cancer [62]. Alternatively, other aberrations, such as mutant forms of RAF or PI3K, manipulate the downstream signaling in cancer through negative feedback loops [63]. Thus, computational charting of EGFR/ErbB signaling may guide the cell-fate decision. Altogether, it is conceivable to speculate that assessing individual ErbB family receptor expression is insufficient for estimating the biological potential of patients with cancer.

A number of studies have reported the association of EGFR and ErbB2 overexpression with advanced stages of bladder cancer. These phenotypes are thought to predict patient survival, as well as the response to chemotherapy [64, 65]. However, these biomarkers were not included as molecular indicators for patients with bladder cancer [66, 67]. This review describes an authentic inconsistency of the efficacy of ErbB receptor expression in predicting the risk of recurrence, progression, and mortality in patients with bladder cancer. To some extent, this incongruity is consistent with the discouraging results of clinical trials focusing on blockading ErbB receptor signaling [38, 40–43].

Factors responsible for the discrepancies in this meta-analysis include long periods of patient recruitment, a wide range of disease statuses (T0-1, T0–4, T2–4), variability in the immunohistochemical assays used, bias from staining scoring, cutoff points, and subjectivity in interpreting results. There are additional contributing factors, namely, the length of followup, strategies for detecting events of interest, and inconsistency in the inclusion of clinical and pathological factors for multivariate analysis [68]. Not surprisingly, different treatment plans may have different effects on clinical outcome, which might not be considered a confounding factor [69]. The risks calculated in our meta-analysis may be overestimated by reporting biases because hazard ratios and 95% CIs were not described when associations were not significant.

5. Conclusions

In conclusion, this meta-analysis has revealed a significant association between ErbB family receptor expression and progression and mortality in patients with bladder cancer even though these findings need to be carefully interpreted. Considering current molecular information, assessing ErbB family coexpression patterns may provide better prognostic information for patients with bladder cancer. Updated clinical trials suggest that more investigations are required to identify effective agents for targeting ErbB signaling of urothelial carcinoma. Systematic reviews and meta-analyses are mandatory before accepting ErbB receptor expression patterns as predictive markers for clinical application.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

The Use of Regenerative Medicine in the Management of Invasive Bladder Cancer

Matthew E. Hyndman,[1] Deborah Kaye,[2] Nicholas C. Field,[1] Keith A. Lawson,[1] Norm D. Smith,[3] Gary D. Steinberg,[3] Mark P. Schoenberg,[2] and Trinity J. Bivalacqua[2]

[1] *Southern Alberta Institute of Urology, University of Calgary, Alberta, Canada T2V 1P9*
[2] *The James Buchanan Brady Urological Institute, Johns Hopkins Medical Institutions, Baltimore, MD 21287-2101, USA*
[3] *University of Chicago Medical Center, Chicago, IL 60637, USA*

Correspondence should be addressed to Trinity J. Bivalacqua, tbivala1@jhmi.edu

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Muscle invasive and recurrent nonmuscle invasive bladder cancers have been traditionally treated with a radical cystectomy and urinary diversion. The urinary diversion is generally accomplished through the creation of an incontinent ileal conduit, continent catheterizable reservoir, or orthotopic neobladder utilizing small or large intestine. While radical extirpation of the bladder is often successful from an oncological perspective, there is a significant morbidity associated with enteric interposition within the genitourinary tract. Therefore, there is a great opportunity to decrease the morbidity of the surgical management of bladder cancer through utilization of novel technologies for creating a urinary diversion without the use of intestine. Clinical trials using neourinary conduits (NUC) seeded with autologous smooth muscle cells are currently in progress and may represent a significant surgical advance, potentially eliminating the complications associated with the use of gastrointestinal segments in the urinary reconstruction, simplifying the surgical procedure, and greatly facilitating recovery from cystectomy.

1. Introduction

An estimated 73,510 people in the United States will be diagnosed with bladder cancer resulting in approximately 14,880 deaths in 2012 [1]. It is the fifth most common cancer in the United States and is responsible for 3% of all cancer deaths. Bladder cancer is 3-4 times more prevalent amongst males than females. White males and females are more likely to be diagnosed with and die from bladder cancer than African American males and females. Disease incidence peaks in the 8th decade of life [2–4]. The percentage of patients with invasive cancer also increases with age. The incidence of invasive bladder cancer in men greater than 70 years old is 3.5% compared to 0.41% amongst 40–59-year-old males [2].

Bladder cancer is hypothesized to occur because of a variety of factors, including carcinogen exposure, radiation, chemotherapy, infection, inflammation, nutrition, genetics, and geography. However, the polygenetic basis of bladder cancer is linked to various genetic mutations. Thelen and Schaeuble were the first authors to describe a familial occurrence of bladder cancer; however, a familial syndrome has not been described [5]. An extensive number of genes have been hypothesized to play a role in bladder cancer etiology and prognosis, including chromosome 9 deletions, RAS gene mutations, P53, and Rb [6, 7]. Some of these mutations are common in many types of bladder cancer, while others are more specific to nonmuscle invasive or muscle-invasive disease. Genetic polymorphisms, including the slow NAT-2 polymorphism and null variant glutathione-s-transferase polymorphism may also play a role in bladder cancer, although the outcome data for these polymorphisms is mixed [8].

Environmental exposures are known causes of bladder cancer. An estimated 20–27% of bladder cancers are associated with industrial exposure. Rubber workers have some of the highest incidence of bladder cancer (RR 1.29, CI 1.06–1.58) [9]. These exposures can occur in a variety of fashions

including through-skin absorption or inhalation. There is a long latency period between environmental exposure and bladder cancer diagnosis, thereby making a specific etiology of bladder cancer difficult to determine [10].

Tobacco smoke is associated with a 2–6 times increase in the relative risk of bladder cancer. Aromatic amines, common in certain dyes and arsenic, a common pollutant, are examples of chemicals considered to cause bladder cancer [9, 11–13]. Infection and inflammation have also been shown to be associated with bladder cancer, especially chronic inflammation with squamous cell cancer and schistosomiasis with squamous and transitional cell cancer [14].

Treatment for bladder cancer varies by disease stage. While low-grade disease can often be treated with local resection and intravesical immunotherapy or chemotherapy, muscle-invasive disease usually requires cystectomy with or without neoadjuvant or adjuvant chemotherapy. Primary systemic chemotherapy is often administered to patients with metastatic disease [15].

2. Radical Cystectomy and Postoperative Morbidity

Since the original report by Whitmore and Marshall [16], surgical extirpation of the bladder has become the gold standard for treatment of muscle-invasive bladder cancer and an important treatment option for some nonmuscle invasive tumors. Bladder reconstruction and urinary diversion have been performed utilizing various gastrointestinal segments, however the vast majority of reconstructive procedures used today still involve either the ileum or colon. While numerous surgical variations using these two bowel segments have been described, three techniques predominate; an incontinent ileal conduit, continent cutaneous diversions, and orthotropic neobladder diversion. The majority of postoperative complications related to direct surgical problems associated with the ureterointestinal anastomosis, bowel anastomosis, infection/abscess, or metabolic disturbances related to urine directly contacting the absorptive gastrointestinal tract [17–19]. Stomal complications are also observed and are linked to peri-stomal ischemia and subsequent fibrosis [20].

Although complication rates for radical cystectomy are believed to be declining, it is still associated with significant morbidity and mortality. Prior to 1990, morbidity and mortality rates were 28–42% and 2.4–15%, respectively. Current morbidity and mortality rates have decreased to 11–68% and 0–3.9%, respectively [21]. Using the Clavien grading system, a recent study by Shabsigh, found that 64% of patients experienced a complication within 90 days of surgery, of which 13% were high grade (grade 3–5) defined as a complication requiring an operative intervention (grade 3) or resulting in significant disability (grade 4) or death (grade 5) [22]. Another study by Stimson et al. found that 26.6% of patients were readmitted within 90 days of a radical cystectomy, of these, 19.7% were admitted within 30 days of surgery. Ileus, pyelonephritis, and urinary tract infections were the most common reasons for early readmission, while pyelonephritis was the most common reason for late readmission [23]. Complication rates were recently found to increase with age, male gender, nonteaching hospitals, and hospital surgical volumes [24].

It is difficult to compare complication rates through time, across different studies, and across different types of urinary diversion. Complication reporting is not standardized and the length of followup varies greatly across studies. However, across studies some complications are more common than others. Gastrointestinal complications, including paralytic ileus, small bowel obstructions, emesis, gastritis, and gastric ulcers are often most common (29%), followed by infections (25%) and wound complications (15%) [22]. Infectious complications range from urinary tract infections (12.8%) to septicemia (9.6%) [25]. Wound complications also pose a significant concern. Roughly 0–15% of patients will have a wound infection and 0–9% of patients may suffer from wound dehiscence [21]. Overall, major complications associated with radical cystectomy and urinary diversions are related to the use of the gastrointestinal tract for urinary reconstruction. Other complications include, but are not limited to blood loss and transfusion, urinary extravasation and leak, infections, deep vein thrombosis and pulmonary embolism, intestinal or anastomotic leak, cardiac complications, metabolic disturbances, strictures, and lymphoceles.

Many of the short and long term surgical complications result from replacement of organs designed primarily for storage and excretion with organs which are specialized absorptive structures. The GI tract, especially the small bowl, is microscopically arranged to maximize absorptive surface area with villi and microvilli. Indeed, reabsorption of urine and solutes may lead to a hyperchloremic metabolic acidosis. While usually subclinical, electrolyte abnormalities are in part dependent on the surface area of the diversion. Therefore, patients with diversions requiring more bowl, such as neobladders are more likely to have electrolyte abnormalities. Other metabolic alterations related to urinary diversion include vitamin B12 deficiency decreased enterohepatic recycling of bile salts, and dysregulation of calcium metabolism. Reabsorption of renal excreted medication also results from interposition of the GI tract within the GU system.

The degree of postoperative morbidity associated with radical cystectomy suggests that there is a genuine opportunity to improve treatment-related outcomes through the adoption of novel and innovative reconstructive technologies.

3. Use of Regenerative Technology in Medicine

Regenerative medicine involves replacing or restoring damaged, absent, or dysfunctional tissues. This emerging field has the potential to impact human disease states such as diabetes [26], Alzheimer's [27], cardiovascular disease [28], and musculoskeletal disorders [29]. Cell-based therapies are primarily focused on restoring function rather than generating new organ structures. Regeneration of complex tissues presents an additional challenge where in addition to restoring cellular function, the 3D tissue structure must also be recapitulated. This structure is dependent on integrating multiple cell types to create supportive vascular, nervous,

lymphatic, and structural tissues. A number of approaches are being actively studied to recreate extracellular matrices and supportive tissues. One technique is to use decellularlized matrices of the desired organ structure and then implant or seed the ultrastructure with cells. Alternatively, porous *de novo* ultrastructures can be synthesized and then seeded with cells or both cells and matrixes using "ink jet" printing technology with some success. The advantage of the later techniques is that tailor-made shapes are possible. A matrix seeded approach was recently utilized by Jungebluth et al. for reconstruction of a trachea for a patient with recurrent bronchial carcinoma using a nanocomposite polymer [30, 31]. The polymer was then seeded with autologous mononuclear cells (MNC) harvested from a bone marrow biopsy. This proof of concept study was successful and at last followup the patient was tumor-free and symptom-free at five months [30]. Likewise, Ott et al. decellularized rats hearts and then repopulated them with neonatal cardiomyocytes and remarkably the repopulated hearts were able to generate contractions [32]. Furthermore, using an animal model, Ross et al. repopulated a decellularized kidney with a mouse embryonic cell line which demonstrated that a decellularized ECM can, in part, direct the differentiation of pluripotent cell lines [33]. Animal studies, investigating whole corpora replacement of decellularized penises, have been successfully repopulated and reimplanted with functionality [34]. Expanding these proofs of principle studies to recellularization of complex human organs is complicated in part by the larger size of human structures. Perfusion and nutrient delivery to the seeded cells is dependent on the distance from the microvasculature. Thicker structures, therefore, require complex reendothelialization and revascularization of the seeded ECM structures. Embryonic development of organelles is not limited by this perfusion factor given that organ growth and vascular growth expands proportionally. The success of the aforementioned tracheal replacement study is in part because of the tubular structure of the organs which therefore limits the perfusion distance. Because of its inherent tubular design, the urinary tract is an ideal system for the application of regenerative medicine.

4. Use of Regenerative Medicine in Urology

Replacement or augmentation of the urinary tract has traditionally been performed utilizing autologous bowel. The use of interposing bowel in the urinary tract significantly increases acute surgical complications and the absorptive properties of bowel counteract the fundamental excretory function of the urinary system. Synthetic substitutes have failed primarily because of scaring, poor compliance, and the predictable development of urinary stone formation [35]. More recently, modifications of small intestine submucosa have shown improved smooth muscle cell regeneration but poor long-term maintenance of capacity [36]. Interestingly, bladder matrices preseeded with cells have improved functional properties and decreased scaring and fibrosis compared to unseeded augments [37]. However, augments act as a supplement to the dysfunctional bladder. Replacing bladder after a radical cystectomy complicates organ restoration because the entire functional organ including the supportive vascular and nervous structures is removed. Seeding with autologous bladder tissue is often complicated by the possibility of seeding malignant cells. Therefore, regenerative tissue replacement in the bladder cancer population requires the synthesis of complex structures that ideally is seeded with autologous cells from a source other than the native urothelium.

5. Neourinary Conduit Program

Regenerative medicine principles have been successfully applied to provide implantable cell-seeded matrices for use in the reconstruction, repair, augmentation, or replacement of laminarily organized luminial organs and tissue structures, such as a bladder or a bladder component, typically composed of urothelial and smooth muscle cell layers [38–42]. Smooth muscle cells (SMC) may be derived from the patient's own tissue, including the bladder, urethra, ureter, and other urogenital tissue. However, there are challenges associated with dependence upon the development and maintenance of cell-culture systems from the primary organ site as the basic unit for developing new and healthy engineered tissues. A malignant organ, such as a bladder with established urothelial carcinoma, or utilizing undifferentiated pluripotent cells is not appropriate for sourcing cells populating neoorgans. However, using alternative sources of differentiated mature cells for seeding synthetic, biodegradable tubular scaffold structures for *de novo* formation of urinarylike neotissue *in vivo* may be a more suitable approach.

With regenerative technologies, stable SMC may be used to seed synthetic, biodegradable tubular scaffold structures. With implantation of these seeded scaffolds, the body can regenerate tubular neourinary conduits composed of urinary tissue, that is, histologically identical to native urinary tissue [39, 43]. The ability to create urologic structures *de novo* from scaffolds seeded by autologous smooth muscle cells will greatly facilitate the translation of urologic tissue engineering technologies into clinical practice.

Using SMC and synthetic scaffolds, complete bladder replacements have been designed and implanted to regenerate a complete, innervated, and pharmacologically intact urinary bladder in animals [44]. A conduit from the ureters to the skin surface addresses the current standard of care while simplifying the surgical procedure and may also provide improved patient outcomes. Tengion's neourinary conduit (NUC) serves as a template to catalyze the regeneration of native-like urinary tissue that can connect the ureters to the skin surface. To ensure native urinary tissue regeneration, a biocompatible and biodegradable scaffold with an extended history of safety and clinical utility is necessary. The broadly used scaffold polylactate-glycolate (PLGA) serves the objective to enhance tissue regeneration and promote neotissue integration into the body when properly seeded with SMCs.

Construction of the NUC is based upon two principal components. (i) *Biomaterials*. The NUC scaffold is composed of PGA polymer mesh fashioned into the required tubular

shape and coated with a 50/50 blend of PLGA copolymer. Specific structural parameters may be modified as needed during the surgical procedure to personalize the application to a patient's needs. The choice of well-established, synthetic, and degradable biopolymers reflects the same requirements for reliability and reproducibility inherent in the choice of these polymers for applications in other bladder-related neoorgans. (ii) *Cells*. Autologous smooth muscle cells (SMC) sourced from bladder or nonbladder tissue may potentially be applied for construction of NUC.

Based on the successful outcomes in experimental conditions using a porcine cystectomy model, Tengion has initiated Phase I clinical trials of NUC constructs in human patients requiring urinary diversion. This Phase I study "Incontinent Urinary Diversion Using an Autologous NeoUrinary Conduit" (http://www.clinicaltrials.gov/ct2/show/NCT01087697) is currently recruiting patients, with the objective of implanting up to 10 patients by the end of 2012. The objective of the study is to evaluate if NUC constructs (made using autologous adipose-derived SMCs in combination with defined degradable biomaterial scaffolds) can form a functional conduit to safely facilitate passage of urine from kidneys subsequent to radical cystectomy. Primary outcome indices over a 12-month postimplantation tim eframe include structural integrity and conduit patency. CT scans will be used to demonstrate that urine may flow safely through the NUC construct. Additional measures of primary outcomes up to 12-months postimplantation include an evaluation of any product- or procedure-related to adverse events. Similarly, secondary outcome indices will include analysis of NUC structural integrity and patency over a 12–60-month postimplantation time frame. CT scan and renal ultrasound will be applied to demonstrate that urine flows safely through the NUC construct up to 60-month afterimplantation. Procedural- and product-related adverse events will also be monitored to 60 months afterimplantation. Finally, the overall safety of the NUC construct will be assessed through evaluation of nonproduct/procedural-related adverse events and patient vital signs.

5.1. Future Directions. The use of tissue engineered (TE) bladders as functionally superior alternatives to enteric urinary diversions, which are currently utilized by urologists to reconstruct the genitourinary tract, offers great promise to patients. However, a number of barriers exist that must be overcome before this technology can be successfully integrated into the clinical arena. Undoubtedly, the establishment of a mature blood supply to TE structures represents arguably the largest of these barriers as poorly vascularized tissue grafts have the propensity to develop scar tissue as a result of hypoxia, leading to decreased bladder contractility, compliance and overall function [45–47]. As such, great efforts to improve the efficiency of neoangiogenesis within TE bladders have been undertaken by scientists in the field. To date, a number of tissue engineering strategies have been employed in both urologic and nonurologic tissue grafts towards this goal. All have been developed based on our increased knowledge of the physiological parameters that regulate and promote blood-vessel formation and stability. Of these, the use of endothelial support cells, vascular promoting growth factors and proangiogenic extracellular matrix properties standout as the most promising novel approaches for facilitating the application of TE bladders into urologic practice.

5.2. Endothelial Support Cells. The development of a mature vascular network within tissue grafts requires extensive communication of endothelial cells with their surrounding microenvironment. Central to this are endothelial support cells known as pericytes. These cells mediate blood vessel formation, maturation, and stabilization through their ability to regulate endothelial cell differentiation and growth via direct cell-to-cell contact and paracrine signaling [48]. Due to this critical support role, it is obvious that strategies to improve pericyte coverage of forming vascular networks hold promise. While the implantation of pericytes, directly on grafts is the most intuitive approach, a reliable source of these cells has not been described [49]. However, recent work by Traktuev et al. has demonstrated the ability to utilize a CD34 positive subpopulation of adipose stromal cells (ASC) to stabilize endothelial cell networks [50]. Within adipose tissue, the authors discovered that this cell population resides perivascular and, like pericytes, is capable of communicating via paracrine signaling to endothelial cells. Interestingly, coculture of these cells with endothelial precursor cells (EPC) *in vitro* leads to stable vascular network assembly. Furthermore, matrigel and confocal microscopy revealed their overlaying localization, highlighting the ability to utilize ASCs to promote stable vascularization. In a subsequent study, these authors demonstrated the superiority of coimplanting both ASC with EPC to improve collagen graft implant vascularization in SCID/NOD mice relative to ASC or EPC alone, portraying the advantages of enhancing endothelial support as a strategy to improve graft neoangiogenesis [49]. To date, this strategy has yet to be employed in improving bladder organogenesis and as such, the implantation of adipose stromal cells into bladder scaffolds warrants further investigation for improving vascularization and prevention of scarring in TE bladders.

5.3. Growth Factors. The extracellular microenvironment represents the medium by which endothelial and support cells interact to orchestrate the complex physiological task of forming new vascular networks. Within this medium, an extensive array of molecular constituents exist which act to drive the necessary signaling pathways between these cells. Most notable, is the vascular endothelial growth factor (VEGF), which has been extensively shown to be crucial in promoting and regulating angiogenesis [51]. Within the endothelial cell microenvironment, VGEF is organized in a precise manner in order to allow for the intricate regulation of blood-vessel tubulogenesis and maturation. Interestingly, with the progress made in the field of nanotechnology it is now possible to design scaffolds for organogenesis which are capable of modeling this complexity [52]. Indeed, micropatterning techniques have been utilized to create scaffolds (hydrogels) embedded with VEGF. These hydrogels are hydrophilic, allowing them to resist growth factor (protein)

absorption as well as nonspecific cell adhesion [53]. Moreover, by introducing collagenase sensitive peptide sequences and covalently immobilizing VEGF into the back bone of the hydrogel, an endothelial cell controlled local release of growth factor can be achieved [53]. Further regulation of growth factor release from this hydrogel can be accomplished through embedding of cell adhesive peptide sequences, such as Arg-Gly-Asp-Ser (RDGS), to control where endothelial cell attachment and therefore, liberation of VEGF occurs [53, 54]. Of note, the use of this growth-factor-imbedded hydrogel has been shown to not only improve endothelial tubulogenesis, but also enhance endothelial cell motility and formation of intercellular contacts [53]. Importantly, the release of growth factors from an elastomeric poly (1,8-octanediol-co-citrate) (POC) scaffold, which has been utilized in tissue engineering of bladder tissue, has also been described [47]. In their study, Sharma et al. developed an elastomeric POC scaffold modified with heparin sulphate that was capable of releasing VEGF, fibroblast growth factor 2 and insulin growth factor 1. The use of this VEGF releasing scaffold resulted in augmentation of scaffold vascularization compared to control when implanted in nude (athymic) rats as demonstrated by an increase in CD31 and von willebrand factor (vWF) immunostaining. Thus, the application of this technology to the development of TE bladders holds great promise in improving their clinical functionality and therefore their use. As this technology improves additional growth factors may be simultaneously released as demonstrated by a recent study by Davies et al. who delivered both VGEF and platelet-derived growth factor (PDGF) through their scaffold to maintain graft angiogenesis *in vivo* [55].

5.4. Extracellular Matrix Properties. Beyond growth factors, many other extracellular matrix (ECM) components contribute to the growth regulation and maturation of forming vascular networks. As such, strategies that mimic the proangiogenic effects of nongrowth factor ECM components have been investigated. Highlighting this is a study conducted by Caiado et al. who characterized the ability of fibrin E, a known byproduct of ECM fibrin degradation, to enhance vasculogenesis and wound healing [56]. These authors demonstrated that culture of EPC in ECM containing fibrin E lead to increased adhesion, proliferation, and acquisition of mature endothelial cell markers *in vitro*. Moreover, when scaffolds enriched for fibrin E were tested against integra scaffold controls in a murine Balb-c/SCID wound healing model, they produced marked increases in neovessel formation and facilitated greater wound closure. As such, it is clear that nongrowth factor ECM components contribute to vascularization of grafted tissue. Determination of the ECM constituents that best promote vascularization of TE bladders and subsequently, constructing bladder scaffolds to incorporate these should be investigated. Furthermore, the physical properties of the ECM that also contribute to vasculogenesis of engineered scaffolds must also be considered in the design of future bladder scaffolds. This is highlighted by the finding that modulating the collagen fibril density within scaffold matrices, which changes the scaffolds stiffness properties, has dramatic effects on endothelial colony forming cell (ECFC) vessel formation *in vitro* and *in vivo* [57]. Interestingly, when ECFC are cultured on matrices with higher collagen fibril density and increased matrix stiffness and transplanted into nude mice, an increase in the average vessel area and total vascular area is seen, relative to lower collagen fibril density matrixes [57]. Hence, the design of TE bladder scaffolds may benefit from incorporating both neoangiogenic promoting ECM components (i.e., growth factors, fibrin, etc.) as well as pro-vasculogenic ECM physical properties.

6. Conclusions

Radical cystectomy is an effective treatment for bladder cancer however, reconstruction of the urinary tract with enteric structures whose primary role is absorption causes significant postoperative morbidity. Recent advances in regenerative medicine coupled with the idea of creating a conduit rather than an entire bladder may represent an alternative to the use of gastro-intestinal tissue for post-cystectomy urinary diversion and therefore decrease surgical and postoperative morbidity and lead to improved postoperative outcomes.

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Review Article

Muscle Invasive Bladder Cancer: From Diagnosis to Survivorship

N. E. Mohamed,[1] M. A. Diefenbach,[1] H. H. Goltz,[2] C. T. Lee,[3] D. Latini,[2] M. Kowalkowski,[2] C. Philips,[1] W. Hassan,[4] and S. J. Hall[1]

[1] *Department of Urology and Oncological Science, Mount Sinai School of Medicine, New York, NY 10029-6574, USA*
[2] *Michael E. DeBakey VA Medical Center, University of Houston-Downtown and Baylor College of Medicine, Houston, TX 77030-4298, USA*
[3] *Department of Urology, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48109-0944, USA*
[4] *Department of Urology, Tawam Hospital, 15258 Al Ain, UAE*

Correspondence should be addressed to N. E. Mohamed, nihal.mohamed@mountsinai.org

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Bladder cancer is the fifth most commonly diagnosed cancer and the most expensive adult cancer in average healthcare costs incurred per patient in the USA. However, little is known about factors influencing patients' treatment decisions, quality of life, and responses to treatment impairments. The main focus of this paper is to better understand the impact of muscle invasive bladder cancer on patient quality of life and its added implications for primary caregivers and healthcare providers. In this paper, we discuss treatment options, side effects, and challenges that patients and family caregivers face in different phases along the disease trajectory and further identify crucial areas of needed research.

1. Introduction

Cancer diagnosis is often perceived as a traumatic event that changes an individual's basic assumptions about the self as effective and powerful, and the world as benevolent, controllable, and predictable [1, 2]. This event is even more devastating when cancer patients undergo extensive surgeries that severely debilitate their body image and their psychological and social well-being. Muscle-invasive bladder cancer (MIBC) provides a powerful, yet understudied example of the impact that cancer diagnosis and treatments may have on patients' emotional, physical, functional, and social adjustment [3].

Previous research in this patient population has primarily been focused on health-related quality of life (HRQOL) as an indicator of posttreatment adjustment [4]. Yet, in addition to decrements in HRQOL and body image, patients with MIBC must often resolve treatment decision-related conflicts and overcome financial, communication, literacy, and healthcare service barriers as they move through different phases of the cancer trajectory (i.e., cancer diagnosis, treatment, and survivorship). Despite treatment-related physical and social limitations, patients need to: (1) balance the uncertainty associated with diagnosis and the risk of recurrence; (2) maintain previous social roles and functions; (3) solve financial issues arising from insurance issues and uncertain employment situations.

In this paper, we will provide a comprehensive overview of: (1) MIBC incidence, mortality rates, and risk factors; (2) MIBC-treatment-related side effects; (3) challenges that newly diagnosed MIBC patients face during treatment decision making, treatment-related side effects, and posttreatment healthcare; (4) indicators of posttreatment adjustment survivorship issues. We will address limitations in the existing literature and discuss future directions.

2. Radical Cystectomy

MIBC may be treated with curative intent by either external beam radiation therapy (with or without chemotherapy) or radical cystectomy, with or without perioperative chemotherapy [5]. In the United States, radical surgery is considered standard of care. Radical cystectomy also remains the treatment of choice for NMIBC refractory to

transurethral resection and intravesical therapy, as well as invasive carcinoma of the prostatic urethra. In men, radical surgery traditionally includes the removal of the bladder, prostate, and seminal vesicles; in women the urethra, uterus, cervix, ovaries, fallopian tubes, and anterior vagina have historically been removed along with the bladder [6–10]. In the recent past, however, strategies to preserve sexual and reproductive function in selected patients have led to prostate capsule and seminal vesicle sparing surgery in men and to vaginal sparing surgeries in women with preservation of the reproductive organs based on the patient's fertility status. Radical cystectomy necessitates urinary diversion using one of three major methods: incontinent cutaneous (e.g., the ileal conduit), continent cutaneous, or orthotopic (e.g., the neobladder) diversion.

2.1. Incontinent Cutaneous Diversion. Incontinent cutaneous diversion optimal in a wide spectrum of patients. Conduits can be created using large or small intestine, but due to the manageable metabolic profile and the ease of the bowel segment, the ileal conduit is performed most commonly. An ileal conduit is an incontinent urinary diversion to the skin, requiring the creation of a cutaneous stoma and use of external stoma appliances to collect urine. An ileal conduit is considered the easiest urinary diversion to be performed surgically. Despite the fact that this is the diversion of choice for patients with hepatic or renal insufficiency or those unable to catheterize or timed void, this method has its drawbacks. Patients reporting early and late complications for ileal conduit range from 18% to 30% [11]. Stoma-related complications are common and have been reported in up to 31% of patients; these include parastomal hernia, urinary leakage, and stomal stenosis [11]. Urinary tract deterioration was found in 35% after 5-year followup and in 50% after 15-year followup. Skin irritations due to direct and continuous contact with urine, as well as constipation were also reported [7, 9].

2.2. Continent Cutaneous Diversion. This method involves creating an intra-abdominal, catheterizable urinary reservoir commonly using up to 60–70 cm of ileum, the entire right colon or a combination of small and large bowel. The continent mechanism varies based on the surgical approach [12]. The continent reservoir requires the use of a catheter to drain urine from the reservoir four to six times per day. Failure to catheterize as frequently as recommended can cause serious medical conditions including acute renal failure, perforation, and infection [13]. Early and late complication rates for continent reservoir range from 3%–7% and from 13%–30%, respectively [14]. Physical morbidity reported after continent urinary diversion includes stenosis, pouch leakage, pouch rupture (1.5% to 4.3%) [13], and incontinence. Overall, 3.2%–7.4% of patients report these complications [15]. Additionally, night-time catheterization may be more bothersome than night-time stoma care associated with ileal conduits [16].

2.3. Orthotopic Diversion. This procedure involves the creation of a large-capacity, low-pressure reservoir from colon or ileum that is connected to the native urethra [11]. Patients learn to void through simultaneous relaxation of the pelvic muscles with coordinated increase in their intra-abdominal pressure (Valsalva maneuver) [12, 16, 17]. Clean intermittent catheterization (CIC) is required to manage urinary retention or periodically for irrigation of excessive mucous [12, 16, 17]. Patients who are not able to catheterize themselves practice timed voiding (since normal bladder sensation and urge are no longer intact). Patients with hepatic or renal insufficiency are generally not eligible for this procedure. The time needed for postoperative recovery and for achieving urinary continence is longer in older than in younger patients [16, 17]. Additionally, patients with prostatic stromal or urethral involvement and many women with bladder neck disease are not eligible for this procedure since the urethra must be removed for adequate cancer control. Daytime continence ranges from 67% to 100% and night-time continence ranges from 45% to 65% [12, 18]. The high-storage capacity and interaction of urine with the absorptive surface of the bowel continent reservoir and neobladder can ultimately cause metabolic disturbances and renal failure [11].

3. Psychosocial Effects of Urinary Diversion

3.1. Ileal Conduit. The emotional, functional, physical, and social impact of radical surgery on HRQOL significantly varies by the urinary diversion form [4, 7, 9, 12]. Studies have shown that ileal conduits are considered the easiest urinary diversion to perform surgically; however, [15] it is associated with significant psychological and practical side effects that can drastically reduce the emotional well-being of the patient [7, 9]. These effects include: (1) changed social roles decreased body image, sexual dysfunction, and barriers due to body image, bother caused by urinary leakage, odor, and frequent stoma care; (2) difficulties in activities of daily living and social activities, such as, bathing and sleeping; (3) barriers to participation in previously pleasurable hobbies and travelling [7, 9, 15]. Studies that examined bladder and bowel cancer patients' coping with ostomy surgeries showed that feelings of shock, hate, disgust, repulsion, embarrassment, devastation, and unacceptance were common [19–21]. Patients' coping with ileal conduit and its consequences may depend largely on the patient's personal and social resources and the meaning and importance placed on body image, sexuality, and urinary control [19–21]. Adequate explanation of the treatment's potential effects on sexual function, urinary control, and body image should be provided by the physician during counseling.

3.2. Continent Reservoir. In patients treated with continent reservoir, the body image, theoretically, is less disturbed and stoma care is generally not required as compared to incontinent diversion, but failure to catheterize as frequently as needed can cause urinary leakage and serious medical conditions [22]. In addition, night-time catheterization may result in reduced amount and quality of sleep [21]. In spite of the reduced impact of a continent reservoir on the patient's body image (compared to ileal conduit), the need

for regular catheterization may limit the patient's activities of daily living and his/her participation in work and social activities. Moreover, dietary modifications to avoid frequent urostomy appliance management, voiding, or catheterization required with incontinent and continent reservoirs may result in increased risk for serious medical conditions including acute renal failure [22]. These complications could further debilitate the patient's QOL and emotional adjustment.

4. Neobladder Diversion

A major advantage of the neobladder is that it preserves body image and may be socially desirable more than the ileal conduit and continent reservoir, as it maintains urethral voiding [3]. Patients with neobladder diversion scored better in a scale assessing posttreatment social and physical activities including bathing, sleeping, and travelling and reported fewer sexual problems and barriers than patients with ileal conduit [4]. Although daytime continence is achieved in almost all patients with neobladder diversion, night-time continence is less likely to be achieved. Results from recent research in other patient populations demonstrated the negative impact of night-time incontinence on increased depression and poor quality of life [23, 24]. Additionally, there is evidence that physical impacts of neobladder diversion on QOL vary by gender [18]. Urinary incontinence and deterioration of sexual function are more common among women compared to men following surgery and orthotopic neobladder [18]. Follow-up clinic assessments should examine emotional wellbeing and sexual function among patients regardless of the type of urinary diversion received.

4.1. Cancer Recurrence. The risk of local recurrence following cystectomy has decreased markedly from about 40% reported in initial reports to 6% to 13% observed in recent findings [23, 24]. Approximately 50% of MIBC patients relapse with distant recurrence [9, 25]. The probability of cancer recurrence is associated with tumor stage, grade, and node status at the time of cystectomy. The high rate of recurrence and the concern for disease progression in patients with MIBC necessitate careful surveillance following cystectomy (i.e., every 3–6 months in the first year, every 6 months in the second year and annually thereafter).

4.2. Posttreatment Self-Care. Patients and primary caregivers (referred to as family caregivers throughout this paper) are required to manage the urinary diversion, which involves the acquisition of new skill sets, based upon the diversion choice. Patients with ileal conduit diversion are instructed in the use of stomal appliances and skin care beginning on the second or third day following surgery [26]. The education continues, however, throughout the first several months after diversion as a variety of stomal supplies are explored to provide the best fit for the individual's changing habitus and activity level. For patients with continent diversions and neobladders, training occurs in the first postoperative visits (i.e., 2-3 weeks postsurgery). At this time, catheters and/or drains are removed as patients become more able to manage their pouch. Patients are then instructed to empty the neobladder (through Valsalva voiding or intermittent catheterization) or continent reservoir (via catheterization) on a timed schedule that will permit an increasing pouch capacity [15, 17].

The American Urological Association (AUA) and the Wound Ostomy Continence Nurse Society (WOCN) Joint Position Statement on the Value of Pre-operative Stoma Marking for Patients Undergoing Urinary Ostomy Surgery recommends that patients scheduled to have cystectomy learn about stoma care and use of stoma appliances prior to surgery when they are less distracted than in the immediate postoperative period as typically implemented in BC health care settings [24]. The Joint Position Statement, however, lacks recommendations for patients undergoing continent urinary diversion [24]. It is certainly advantageous for patients with planned continent diversions to gain greater insight into the postoperative management of their diversion prior to treatment. Informing the patient and the family caregiver about treatment effects, emphasizing the importance of timed voiding and self-catheterizing, and providing the skills needed for postsurgical health care before treatment may reduce the risk of posttreatment complications related to voiding habits and enhance treatment decision making and posttreatment QOL.

4.3. Treatment Decision and Decision-Related Outcomes. There is no consensus on the best diversion choice for patients presenting with MIBC as each of the three previously mentioned diversion methods has clear advantages and disadvantages as discussed in the previous sections [4, 7, 9, 11, 13]. Current data on the long-term efficacy of various treatment options are sparse, and support for these techniques is derived primarily from their short- to intermediate-term functional results [11]. This situation can potentially increase the difficulty in making treatment decisions as newly diagnosed patients must consider incomplete data about treatment options and side effects, as well as contradictory treatment recommendations from their physicians. Results of semistructured patient interviews revealed that patients often lack full information about treatment options and related side effects, depending on their physician for this information, resulting in decreased participation in decision making [26]. Patients need to understand how differing treatment options might influence their QOL in the short and long term when making treatment decisions. In addition, physicians need to be able to objectively communicate treatment and side-effect information to patients and facilitate integration of this information into their patients' decision-making process [27, 28]. Incomplete information about treatment, its risks and benefits, and its impact on lifestyle and relationships may cause patients to make treatment decisions incompatible with their values and preferences and lead to dissatisfaction with treatment and a decline in patient care [27, 28]. When compared to other cancers, literature on BC treatment decision making is scarce.

5. Indicators of Posttreatment Adjustment: Health-Related Quality of Life

Most of the research on the adjustment to a diagnosis of BC and its treatment has focused on examining perceived HRQOL following treatment. Perceived HRQOL refers to patients' appraisal of and satisfaction with their current level of physical, emotional, and social functioning as compared to what they perceive to be possible or ideal [28]. In the case of patients subjected to radical cystectomy and urinary diversion, posttreatment QOL and skills needed for post-treatment health care could significantly affect the patient's choice of the type of urinary diversion form as no significant differences have been determined between the three urinary diversion forms in terms of cancer control and survival rate [11, 29, 30]. In spite of the increased research focus on QOL following cystectomy and urinary diversion, little is known about the psychosocial impact of treatment for MIBC.

5.1. Measures of Quality of Life Following Invasive Bladder Cancer Treatment. HRQOL among MIBC patients is commonly assessed using generic measures, disease-specific measures, or both. Generic HRQOL measures typically assess emotional and functional well-being; examples include *the profile of mood state*, the medical outcome study's 36-item *short form health survey* (sf-36), and *the quality of wellbeing scale* [13, 31]. Although generic measures allow for easy comparisons of HRQOL between different diseases and treatment types and are commonly used among cancer patients, they are not sensitive to bladder cancer treatment-specific side effects [9]. This lack of sensitivity to treatment-specific side effects may limit their ability to detect important aspects of BC-related HRQOL and potential changes in posttreatment HRQOL [9].

Disease-specific HRQOL measures are used to assess broad cancer treatment side effects or BC-specific effects (see Table 1 for more examples). Measures assessing general cancer effects include The European Organization for Research and Treatment of Cancer (EORTC), *Quality of Life Questionnaire version 3.0 QLQ-C30* (EORTC QLQ-C30), and *The Functional Assessment of Cancer Therapy-General* (FACT-G) [13, 31]. Those assessing MIBC effects include the EORTC's Quality of Life Questionnaire QLQ-BLM30 (EORTC-QLQ-BLM30) and *The Functional Assessment of Cancer Therapy-Bladder* (FACT-BL) [13, 31]. Although, these scales are more sensitive to changes in HRQOL following BC treatment, they lack sensitivity to specific psychological impact of treatment and gender differences in treatment side effects and differences in side effects between urinary diversion options [9]. Additionally it is important to address patients' beliefs (i.e., identity, timeline, cure beliefs, consequences) and expectations regarding their treatment outcomes [32] Applications of the self-regulation theory (SRT) in cancer research showed patient illness perceptions and expectations that can influence posttreatment emotional adjustment directly and indirectly by influencing patients' control beliefs and coping strategies [1, 2]. There is a need for research and outcomes measures that address these issues among patients with MIBC.

5.2. Quality of Life Research Outcomes in Invasive Bladder Cancer Patients: Methodological Issues. The international medical literature on QOL following MIBC treatment focused on examining global QOL as well as disease-specific HRQOL (see Table 2). Although no superiority of any of the three urinary diversion forms in terms of overall QOL was found, patients with ileal conduits reported more problems with stomal issues, such as, leakage and bother with odor, less sexual activities, less satisfaction with treatment outcomes, greater distress with urine leakage, and lower body image compared to patients treated with cystectomy and continent reservoir [6, 33–38]. Although, in some studies, patients with neobladders scored better in physical function and body image than those with ileal conduit and continent reservoirs, they were also more likely to report a general decrease in urinary function and higher incontinence rates than those with ileal conduit and continent reservoirs [37, 39–42]. In spite of the preponderance of studies that examined QOL in MIBC patients, the methodological limitations of these studies may limit the generalizability of their outcomes [4, 29, 43]. These methodological limitations include lack of longitudinal and prospective research that examined changes in posttreatment HRQOL and the paucity of randomized controlled studies testing the validity and reliability of tools used in the assessment of HRQOL [4, 29, 43]. Given these methodological limitations, questions remain as to the nature of the information HRQOL research provides and whether this information is of clinical use during preoperative consultations with patients. Table 2 depicts examples of studies and variations in HRQOL measures in patients undergoing radical surgery.

6. Indicators of Posttreatment Adjustment: Impact of Age and Overall Health

After age 30, most organ systems begin exhibiting progressive changes in physiological functioning, even in the absence of disease [39]. These changes result in declines in cognitive, emotional, physical, and social functioning status and affect roughly 60% and 76% of adults ages 65–79 and 80 years and older, respectively. A recent cohort study of older BC patients found that comorbidities, such as, heart disease, stroke, arthritis, and urinary, hearing, and vision problems increased in prevalence with age in this population [40].

Current health status and functioning are often key factors in determining eligibility for cystectomy and other MIBC treatments. For example, much older adults are less likely to undergo radical cystectomy. Rates of 55% have been reported for adults ages 55–59 years compared to 24% and 16% for those ages 75–79 years and 80–84 years [40]. Additionally, when older adults are offered cystectomy, they are typically offered ileal conduits and not neobladders [39]. They are also less likely to receive optimal doses of chemotherapeutic agents [39].

Thus, older adults diagnosed with MIBC face twin challenges to maintaining good quality of life. These include (1) addressing the effects of physiological aging and comorbidities, which may reduce their eligibility for and

Table 1: Quality-of-life instruments. (With permission from Lee et al. [44]).

Instrument	Institution	Domains	Validated	Reliable	Cancer specific	Bladder cancer specific	No. of items
BCI [33, 45]	University of Michigan	Urinary Bowel Sexual function	Yes	Yes	Yes	Yes	34
FACT-VCI [46]	Vanderbilt University	See FACT-G Urinary Bowel Sexual function	Yes	Yes	Yes	Yes (limited tocystectomy)	45
QLQ-BLM 30 module [47]	EORTC	Urinary Bowel Sexual function	Ongoing studies	Ongoing studies	Yes	Yes (muscle-invasive disease)	30
QLQ-BLS 24 Module [45]	EORTC	Urinary Bowel Sexual function	Ongoing studies	Ongoing studies	Yes	Yes (nonmuscle-invasive disease)	24
FACT-BL [48]	FACIT	See FACT-G Limited urinary Limited bowel Limited sexual function	Ongoing studies	Ongoing studies	Yes	Yes	39
FACT-G [49]	FACIT	Physical Social Emotion Function	Yes	Yes	Yes	No	27
QLQ-C30 [47]	EORTC	5 Functional scales 3 Symptom scales 1 Global health/QOL scale	Yes	Yes	Yes	No	30
SF-36 [34]	RAND	8 Domains including Physical Mental Social function Emotional	Yes	Yes	No	No	36

BCI: Bladder Cancer Index; EORTC: European Organization for the Research and Treatment of Cancer.
FACIT: The Functional Assessment of Chronic Illness Therapy.
FACT-BL: Functional Assessment of Cancer Therapy—Bladder Cancer (Extension of the FACT-G + 12 additional bladder cancer-specific items including incontinence, diarrhea, body image, sexual function, and stoma care).
FACT-G: Functional Assessment of Cancer Therapy General.
FACT-VCI: Functional Assessment of Cancer Therapy—Vanderbilt Cystectomy Index (Extension of the FACT-G + 17 additional bladder cancer and treatment specific items).
QLQ-C30: EORTC Quality-of-Life Core Questionnaire.
QLQ-BLM30: EORTC Quality-of-Life Core Questionnaire—Bladder Cancer Muscle Invasive (Extension of the QLQ-C30 + 30 additional bladder cancer-specific items; http://www.eortc.be/home/qol/files/SCManualQLQ-C30.pdf).
QLQ-BLS24: EORTC Quality-of-Life Core Questionnaire—Bladder Cancer Superficial (Extension of the QLQ-C30 + 24 additional bladder cancer-specific items; http://www.eortc.be/home/qol/files/SCManualQLQ-C30.pdf).
RAND Corporation: **R**esearch **AN**d Development.
SF-36: Medical Outcomes Study 36-Item Short Form.

tolerance of invasive BC treatments, such as, cystectomy or chemotherapy; (2) addressing the impact of treatment-related comorbidities and postsurgical complications. Various studies report perioperative morbidity rates of 30–60% among older adults [39]. However, chronological age may not be predictive of the severity of such complications. One study found differences in overall complication rates for older adults receiving neobladders (versus ileal conduits) while another found no differences in daytime urinary continence [39]. Nighttime continence was almost 100% at 5-year followup for adults 50 years or younger and 90% for those over age 60. Beyond urinary continence, few studies have focused on other quality of life issues among postcystectomy older adults, such as, sexual functioning, psychological functioning, social relationships, body image, and gender roles.

7. Indicators of Posttreatment Adjustment: Psychological Distress

Cancer diagnosis may be considered the archetypal experience of loss and is a continuous threat to the patient's

Table 2: Quality of life in patients treated with continent and incontinent urinary diversion.

Author (Year)	Location	Sample size			Scale(s) used	Principal findings
		IC	CCD	NB		
Prospective longitudinal studies of quality of life in patients treated with urinary diversion						
Hardt et al. [8] (2000)	Germany	24	20		SF-36, Self-design	Sexual fxn similar, all SF-36 domains returned to baseline by 1yr except physical fxn. Note: 75% would choose the same diversion
Somani et al. [50] (2009)	UK	29		3	SEIQoL-DW, SWLS, EORTC QLQ-C30	"Family", "health", "relationships", and "finance" identified as biggest contributors to QoL
Cross-sectional studies of quality of life in patients treated with urinary diversion						
Boyd et al. [35] (1987)	USA	87	85		BDI, POMS, Self-design	Preoperative education important for adaptation. CCD: more likely to be sexually active
Mansson et al. [51] (1988)	Sweden	40	20		Self-design	IC: more problems with stomal issues such as leakage + odor
Bjerre et al. [52] (1995)	Denmark	29		38	Self-design	NB with greater incontinence, but IC with greater distress from leakage and lower body image
Gerharz et al. [36] (1997)	Germany	131	61		Self-design	Similar coping strategies, social support. CCD: Better global QOL and fewer stomal issues
Okada et al. [6] (1997)	Japan	63	74		Self-design	CCD: less local stoma problems; greater satisfaction
Hart et al. [37] (1999)	USA	25	93	103	4 Self-report questionnaires	Overall, high QOL in all groups. IC: worse social function
Kitamura et al. [53] (1999)	Japan	36	22	21	EORTC QLQ-C30	Similar overall QOL between groups. IC: more trouble with public restrooms + travel
Fujisawa et al. [39] (2000)	Japan	20	36		SF-36	Similar QOL between diversion types
McGuire et al. [54] (2000)	USA	38	16	38	SF-36	IC: statistically lower mental well being than population-based norm.
Conde Redondo et al. [55] (2001)	Spain	6		27	Self-design	IC: greater distress with urine leakage + lower body image
Hobisch et al. [56] (2001)	Austria	33		69	EORTC QLQ-C30	NB better across all domains
Dutta et al. [57] (2002)	USA	23		49	SF-36, FACT-G	NB marginally better on several domains
Cross-sectional studies of quality of life in patients treated with urinary diversion						
Hara et al. [58] (2002)	Japan	37		48	SF-36, Self-design	Equivalent in all domains of SF-36
Mansson et al. [59] (2002)	Sweden		35	29	FACT-BL, HADS	NB: more problems with incontinence
Protogerou et al. [60] (2004)	Greece	58		50	EORTC QLQ-C30	Included matched 54 patient control group. IC with greater urine odor and day and nighttime leakage than NB or controls.

Table 2: Continued.

Author (Year)	Location	Sample size			Scale(s) used	Principal findings
		IC	CCD	NB		
Allareddy et al. [61] (2006)	USA	56	26		FACT-BL	Compared to intact bladder ($n = 177$): Sexual fxn lower in individuals undergoing cystectomy. Similar QOL between diversion groups.
Kikuchi et al. [40] (2006)	Japan	20	14	15	FACT-BL	Overall QOL similar between groups. NB: worse urinary control, better body image
Gilbert et al. [33] (2007)	USA	66		122	BCI	Urinary, bowel and sexual differences exist by diversion type. NB may have worse urinary fxn
Saika et al. [62] (2007)	Japan	56	31	22	EORTC QLQ-C30, Satisfaction	Assessment of patients 75+ yrs old: Similar QOL between diversion types, including 31 with ureterocutaneostomy.
Harano et al. [63] (2007)	Japan	20		21	SF-36, Self-design	Similar QOL between diversion types
Philip et al. [64] (2009)	United Kingdom	24		28	SF-36	Overall QOL similar NB: Better physical function
Sogni et al. [65] (2008)	Italy	18		16	EORTC: QLQ-C30, QLQ-BLM30	Assessment of patients 75+ yrs old: Similar QOL between diversion types. NB: 56% and 25% daytime and nighttime complete continence rates
Hedgepeth et al. [19] (2010)	USA	89		144	BCI, EORTC BIS	Included bladder-intact comparison group ($n = 112$); NB: Lower urinary function. Body Image is similar between NB and IC.

Note. Urinary Diversion Type: IC: Ileal conduit, CD: Continent cutaneous diversion, NB: Orthotopic Neobladder.
"Self-design": questionnaires developed specifically for the study.
SF-36: Medical Outcomes Study 36-Item Short Form.
HADS: Hospital Anxiety and Depression Scale.
EORTC BIS: European Organization for the Research and Treatment of Cancer Body Image Scale.
SEIQoL-DW: Schedule for Evaluation of Individual Quality of Life-Direct Weighting.
SWLS: Satisfaction With Life Scale.
BDI: Beck Depressive Inventory.
POMS: The Profile of Mood States.

life. Cancer does not only signify an existential plight; it may arouse extreme negative emotions among those who are affected [2]. Accordingly, patients suffering from cancer or other life-threatening diseases usually need to change goals and disengage from many commitments in order to cope with the multiple medical, social, psychological and financial implications of their conditions. Although previous research among certain cancer populations has examined psychological distress, depression, coping, and emotional adjustment in both newly diagnosed patients and survivors, these issues are essentially unexplored in patients with MIBC [2, 41].

8. Survivorship Issues of MIBC Patients and Their Family Caregivers

Examining needs among cancer survivors and their family caregivers revealed different areas of unmet needs [2, 42]. These include: psychological needs (i.e., needs for help with emotional issues), health system and information needs, physical and social daily living needs, emotional support, and interpersonal communication needs [66]. Family caregivers of cancer survivors often feel unprepared for the cancer experience, have limited knowledge about what to expect regarding cancer and treatment, and receive little guidance

and support from the oncology team about how to provide care and support to the patient during and following treatment [67, 68]. Moreover, among younger and middle-aged family caregivers, worries about job loss, other family responsibilities, limited social activities of daily living, and reduced productivity add to the burden of care giving [66, 68, 69]. Most family caregivers of BC patients, however, are older. This population is especially vulnerable to the emotional and physical impact of caregiving, particularly in the face of their own health problems and limited economical and social resources when compared to younger family caregivers. Older family caregivers are more likely to have comorbid diseases, live on a fixed income, and have a limited social network while they provide care and support for the patient. As a result, older family caregivers are more likely to become fatigued from interrupted night sleep and the emotional burden of caregiving. Poor physical condition, increased depressive symptoms, and greater mortality are risks encountered among older family caregivers [69, 70]. To date, no study has examined determinants of psychological adjustment and needs of BC patient and their family caregiver across various types of urinary diversion.

9. Implications for Practitioners and Researchers

Given the side effects of radical surgical treatment for MIBC, there is a strong need for consistent and clear communication between care providers and patients/family caregivers. Increasing patient awareness about treatment options, associated risks and benefits, and short-and long-term treatment effects may aid the patient's decision making and postoperative preparedness. More consistent use of HRQOL instruments in urologic practice, assessment of family caregivers support, and pre- and postsurgical counseling can facilitate patients' coping and satisfaction. More research is needed to examine both patient and family caregiver needs in the context of age, gender, and diversion type. Identifying these needs preoperatively is vital in providing necessary support to reduce care burden and to ultimately guide the design and evaluation of future psychosocial interventions.

10. Conclusions

MIBC presents challenges to both the patient and the family caregiver at each step along the disease trajectory; from diagnosis to treatment to survivorship. Patients diagnosed with MIBC are expected to live for the rest of their lives with the physical emotional and social consequences and practical implications of their treatment choices. Consequently, it is important for patients and the family caregivers to understand how different treatment options might influence their quality of life in the short- and the long-term when making these decisions, particularly concerning the urinary diversion. However, the optimal form of urinary diversion is uncertain. Patients and family caregivers need to evaluate imperfect data from various studies and balance perceived benefits of a diversion with the reality of reduced quality of life and added burden of health. The difficulty of making a treatment decision is further compounded by the small window of time between diagnosis and treatment which make seeking 2nd and 3rd experts' opinions a challenge. Despite the gravity of treatment and the associated side effects and posttreatment care, no study has examined the needs of MIBC survivors and their family caregivers. A proactive approach should be undertaken to prepare the patient and the family caregiver for posttreatment health management, prior to therapy. Such action may facilitate patient decision making and also assist both the patient and family caregiver in coping with difficulties and challenges that arise after treatment.

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Review Article

A Decade of FGF Receptor Research in Bladder Cancer: Past, Present, and Future Challenges

Erica di Martino,[1] Darren C. Tomlinson,[2] and Margaret A. Knowles[1]

[1] *Section of Experimental Oncology, Leeds Institute of Molecular Medicine, St James's University Hospital, Leeds LS9 7TF, UK*
[2] *Institute of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, UK*

Correspondence should be addressed to Erica di Martino, e.dimartino@leeds.ac.uk

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Fibroblast growth factors (FGFs) orchestrate a variety of cellular functions by binding to their transmembrane tyrosine-kinase receptors (FGFRs) and activating downstream signalling pathways, including RAS/MAPK, PLCγ1, PI3K, and STATs. In the last ten years, it has become clear that FGF signalling is altered in a high proportion of bladder tumours. Activating mutations and/or overexpression of *FGFR3* are common in urothelial tumours with low malignant potential and low-stage and -grade urothelial carcinomas (UCs) and are associated with a lower risk of progression and better survival in some subgroups. *FGFR1* is not mutated in UC, but overexpression is frequent in all grades and stages and recent data indicate a role in urothelial epithelial-mesenchymal transition. *In vitro* and *in vivo* studies have shown that FGFR inhibition has cytotoxic and/or cytostatic effects in FGFR-dependent bladder cancer cells and FGFR-targeted agents are currently being investigated in clinical studies for the treatment of UC. Urine-based tests detecting common *FGFR3* mutations are also under development for surveillance of low-grade and -stage tumours and for general population screening. Overall, FGFRs hold promise as therapeutic targets, diagnostic and prognostic markers, and screening tools for early detection and clinical management of UC.

1. Urothelial Carcinoma: Clinical Management and Challenges

Bladder cancer is a common malignancy with over 70,000 estimated new cases and 14,000 deaths per year in the USA alone [1]. In western countries, around 90% of bladder tumours are transitional cell carcinoma, with rare cases of squamous cell carcinoma and adenocarcinoma [2]. Bladder tumours are classified using the TNM classification system [3] according to their invasiveness (stage Ta: confined to the urothelium; T1: invading the lamina propria; T2: invading the muscular layer; T3: invading the submuscular layers; T4: disseminating to other organs) and their differentiation state (1973 WHO grading system: grade 1, 2, or 3 [4]; 2004 WHO grading system: PUNLMP: papillary urothelial neoplasm of low-malignant potential, low grade: well-differentiated neoplasms, high grade: poorly differentiated neoplasms [2]). At presentation, the vast majority of urothelial carcinomas (UC) (~70%) are low-grade superficial papillary tumours with a relatively benign prognosis. Their conventional treatment involves surgical resection and intravesical chemo- or immunotherapy [5]. One of the major challenges in the management of these tumours is their propensity to recur, therefore requiring frequent and often life-long surveillance with cystoscopy and urine cytology. This, coupled with a relatively long life expectancy (5-year survival rate >90%), makes superficial bladder cancer the most expensive and time-consuming malignancy to treat [6, 7]. A minority of superficial tumours (~15%) will eventually progress to become invasive. Despite treatment with radical cystectomy, radiotherapy, and adjuvant or neoadjuvant chemotherapy, newly diagnosed invasive bladder tumours and superficial tumours that have progressed to invasion often metastasize and the 5-year survival rate is poor (<40%) [8].

Currently there are no validated prognostic molecular biomarkers to guide the clinical management of UC. Crucial therapeutic decisions are based on risk tables that include tumour size and number, and previous history [9], in

addition to histopathological criteria, which are often limited by inter- and intraobserver variability and have relatively low reproducibility [10, 11]. Overall, UC management would greatly benefit from rapid cost-effective and noninvasive methods for screening and surveillance, and reproducible and objective molecular biomarkers to predict the risks of recurrence and progression so that more aggressive therapeutic regimes and intensive monitoring could be focussed on patients at higher risk. Furthermore, novel therapeutic approaches and related predictive biomarkers are needed, for use alone or in combination with conventional treatment, to reduce recurrence rate and progression of superficial tumours and prolong survival and quality of life in patients with invasive and metastatic tumours.

2. Structure and Function of Fibroblast Growth Factor Receptors

In humans, fibroblast growth factors are a family comprising 18 growth factors (FGFs) and 4 FGF-homologous factors (FHFs), many of which play a crucial role during both normal physiological processes, such as embryogenesis, development, and wound healing, and a range of pathological conditions [12–14]. The effects of FGFs are mediated by a family of four fibroblast growth factor receptors (FGFR1–4). FGFRs are transmembrane glycoproteins with a conserved structure comprising an extracellular portion with two to three immunoglobulin-like domains (IgI–III), a transmembrane domain, and an intracellular split tyrosine-kinase domain. IgI and IgII are separated by a short negatively charged serine-rich segment, termed the "acid box", followed by a heparin-binding domain with high affinity for heparan sulphate proteoglycans (HSPGs) [12, 13]. IgI and the acid box are thought to have an auto-inhibitory function [15], while IgII and IgIII bind to FGFs in association with HSPGs. FGF binding to the monomeric receptor triggers its dimerization and subsequent transphosphorylation of tyrosine residues in the kinase domain. This initiates a phosphorylation cascade involving a number of docking proteins, resulting in signalling through various downstream pathways, including PLCγ1, RAS-MAPK, and PI3K and STATs [16]. These pathways regulate a variety of cellular functions, including proliferation, migration, and differentiation [16].

Affinity for specific FGFs varies between receptors [17, 18] and a further layer of complexity is added by the fact that FGFRs are subject to alternative splicing, generating isoforms with different ligand-binding specificity in different cell lineages. For example, two alternative isoforms of FGFR3, denoted "b" and "c" are produced by mutually exclusive splicing of exon 8 and exon 9, affecting IgIII [19]. FGFR3b is expressed in epithelial cells and has high affinity only for FGF1. FGFR3c is expressed in cells of mesenchymal origin and has affinity for FGF1, FGF2, FGF4, and other FGFs [17, 19]. Similarly, an alternative FGFR1 isoform, denoted FGFR1β, lacking the IgI domain and with increased affinity to FGF1 and heparin compared to FGFR1α, has been described [20]. Secreted isoforms of FGFRs have also been reported [21, 22].

A fifth FGF receptor has been described [23]. FGFR5 is homologous to the other four receptors in the extracellular portion, but lacks the tyrosine-kinase domain, which is replaced by a short histidine-rich sequence. FGFR5 is therefore regarded as a decoy receptor, which can inhibit signalling by binding and sequestering FGFs [24].

3. Aberrant FGF Signalling in Urothelial Malignancies

3.1. FGFR3 Alterations in Urothelial Tumours

3.1.1. Activating Mutations. Somatic activating mutations of *FGFR3* were first described in UC over ten years ago [25, 26]. Subsequent larger studies established that *FGFR3* mutations occur in around 50% of both lower and upper urinary tract tumours and these cluster in three distinct hotspots in exons 7, 10, and 15 [27–32] (Figure 1). The most common mutations in exon 7 and 10 are S249C (~61%), Y375C (~19%), R248C (~8%), and G372C (~6%), with others occurring at very low frequencies (<2%). Mutations in exon 7 and 10 create a cysteine or glutamic acid residue in the proximal extracellular region of the receptor. The abnormal residues form either disulfide or hydrogen bonds between adjacent monomer receptors, favouring ligand-independent dimerization, transactivation, and signalling [33–35]. Mutations in exon 15 are rarer, with a frequency of around 2%, and they all involve the lysine residue at position 652, which is mutated to glutamic acid, glutamine, threonine or methionine. They are thought to induce a conformational change in the kinase domain resulting in ligand-independent receptor activation and signalling [36]. They have also been shown to alter FGFR3 cellular localization, inducing aberrant signalling from the endoplasmic reticulum [37].

FGFR3 mutations are frequent in benign skin tumours [43] and have been reported at low frequency in cervical carcinoma [25] and multiple myeloma [44], but are absent in other solid cancers [45, 46], suggesting a tissue-specific role. Interestingly, the relative frequency of different *FGFR3* mutations is dependent on the tumour type, with multiple myeloma mostly showing changes in the tyrosine-kinase domain [44], and bladder and cervical tumours mainly exhibiting mutations of the extracellular region. Furthermore, while S249C is by far the most frequent mutation in bladder (Figure 1) and cervical tumours [25], mutation of the adjacent codon (R248C) is the commonest change found in benign skin tumours [43]. It is currently unclear whether the spectrum, frequency and tissue specificity of *FGFR3* mutations is determined by exposure to specific carcinogens or by their functional significance. The role of smoking and occupational exposure to polycyclic aromatic hydrocarbons in determining the frequency or the type of *FGFR3* mutations in UC has been excluded [47, 48]. However, the limited range of hotspot codons in the receptor makes this a difficult target to study from the epidemiological viewpoint and the possibility of small influences of these exposures cannot be excluded without much larger studies. We have recently shown a correlation between the level of ligand

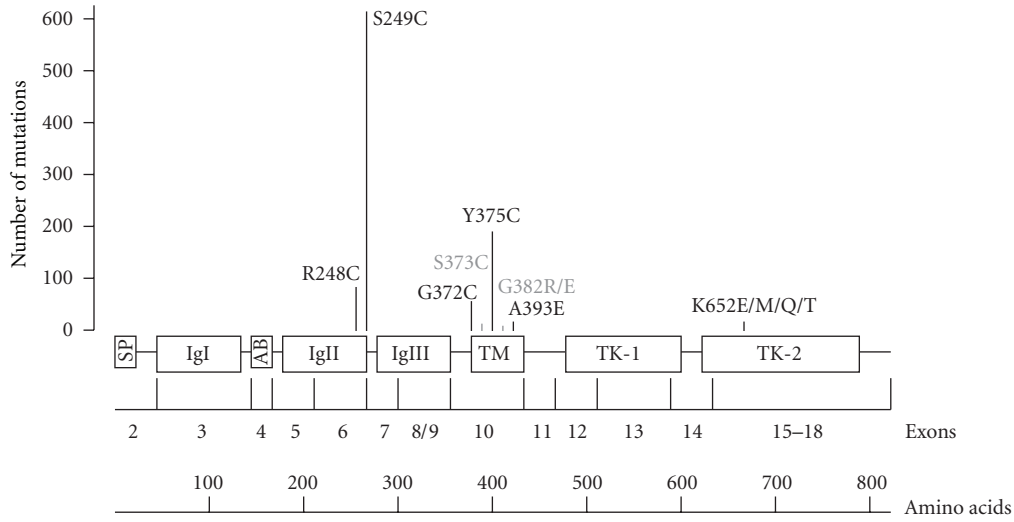

Figure 1: Schematic representation of human FGFR3 protein and corresponding *FGFR3* coding exons. Exon numbering based on Tomlinson et al. [38]. Type and total number of reported mutations are based on data pooled from 11 studies [25–29, 31, 32, 39–42], including a total of 1898 bladder tumours. SP: signal peptide; IgI–III: immunoglobulin-like domain; AB: acid box; TM: transmembrane domain; TK; tyrosine-kinase domain.

independence, signalling activation, and phenotypic consequences of different *FGFR3* mutations expressed in normal urothelial cells and their frequency in UC, suggesting that the spectrum of *FGFR3* mutations in bladder tumours may relate to selection for their potency [49]. We also highlighted cell-type-dependent phenotypic and signalling consequences of specific *FGFR3* mutations which may explain the differences in the relative frequencies between tumour types [49].

During urothelial transformation, *FGFR3* mutations are thought to occur early, as they are reported in flat urothelial hyperplasia, a preneoplastic lesion [50]. Furthermore, *FGFR3* mutations are extremely common in the most benign bladder lesions (low malignant potential neoplasms and urothelial papillomas) and low-grade and -stage tumours (PUNLMP; TaG1), reaching frequencies over 80% in these subgroups [27, 28, 39]. This evidence points to an overall "benign" effect of *FGFR3* mutation in the bladder. Interestingly, all somatic mutations reported so far in UC have been previously described as germline mutations in skeletal dysplasia syndromes, due to the important role of FGFR3 in regulating chondrocyte proliferation and differentiation [51].

3.1.2. Overexpression and Alternative Splicing. Overexpression of wild-type FGFR3 due to t(4; 14) translocation, which places *FGFR3* in the proximity of the regulatory region of the IgH locus, is common in multiple myeloma [44]. Such rearrangements have not been described in bladder cancer. However several reports have examined FGFR3 protein expression in bladder carcinomas, describing an increase in a high proportion of tumours, particularly in the low-grade and low-stage subgroups [32, 40, 52–54]. Two recent investigations have examined the correlation between mutation status and protein expression, showing that up to 85% of the mutated tumours also have increased protein levels [32, 40]. Overexpression of FGFR3 was also detected in around 40% of wild-type tumours, and this was more common in invasive cases. Overall, around 80% of non-invasive and 54% of invasive UC have dysregulated FGFR3 either through mutation, overexpression or both [32]. Therefore, FGFR3 plays a key role in both superficial and invasive disease. However, while superficial tumours tend to exhibit activating mutations of *FGFR3*, often accompanied by protein upregulation, invasive tumours more commonly show upregulation of wild-type FGFR3. At this stage, it is not clear whether this difference reflects differential downstream signalling consequences of wild-type and mutant receptors or the different molecular pathways through which these tumours develop. The molecular mechanisms driving FGFR3 protein overexpression in UC are also still largely unknown, although a recent study has shown that FGFR3 expression in urothelial cells is regulated by two microRNAs (miR-99a/100), which are often downregulated in UC, particularly in low-grade and low-stage tumours [55].

Overexpressed FGFR3 could contribute to tumour development by either ligand-dependent or independent mechanisms. FGF levels are often increased in urine and tumour tissue of bladder cancer patients [56, 57]. Parallel overexpression of FGFs and FGFRs could therefore result in upregulated FGF signalling. It is also speculated that overexpression of the wild-type receptor may favour ligand-independent dimerization and signalling due to the close physical proximity of FGFR3 monomers on the cell surface. Overexpression of FGFR3 would be particularly deleterious if accompanied by a switch to alternative isoforms with different FGF affinity profiles, which would allow tumour cells to activate FGF signalling in response to a greater number of substrates. A switch from the epithelial FGFR3b to the mesenchymal FGFR3c isoform, with broader ligand affinity has been described in bladder cancer cell lines [38]. However, as FGFR3c was not detected in a panel of 76 bladder carcinoma [25], the role of FGFR3 isoform switching in UC *in vivo* is

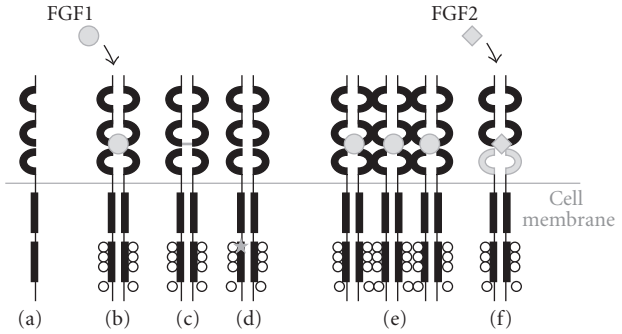

Figure 2: Mechanisms of physiological (a)-(b) and pathological (c)–(f) activation of FGFR3. (a) Monomeric inactive receptor; (b) Ligand-dependent dimerization and activation; (c) Ligand-independent dimerization and activation induced by mutation of the extracellular portion; (d) Ligand-independent activation due to mutations of the tyrosine-kinase domain; (e) Upregulation of signalling due to receptor overexpression; (f) Alteration of splicing favouring isoforms with broader ligand specificity.

still unclear. The different mechanisms of FGFR3 abnormal activation in bladder cancer are summarized in Figure 2.

3.2. FGFR1 Alterations in Urothelial Tumours. In many malignancies FGFR1 has been implicated as an oncogene whose expression or genetic arrangement is altered compared to normal tissue [58–63]. In mouse models of prostate and breast carcinoma, FGFR1 activation *via* an inducible regulation system accelerated progression to malignancy [64–67]. Furthermore, FGFR1 signaling was shown to contribute to the survival of a breast cancer cell line, indicating FGFR1 as potential therapeutic target [63]. More, recently it has been shown that FGFR1 is overexpressed in bladder cancer [68]. Interestingly, FGFR1 expression was increased in both noninvasive and invasive tumours. In light of the changes in FGFR3 splicing observed in UC cell lines [38], FGFR1 splicing was examined. This revealed an altered ratio of FGFR1 α and β splice variants, with increased expression of the β isoform, lacking the IgI domain. The increased expression of this splice variant was significantly associated with tumour stage and grade and caused an increased sensitivity to FGF1 and enhanced downstream signalling [69]. Overall these studies demonstrate that FGFR1, *via* overexpression or altered splicing, may play a key role during bladder tumour development and/or progression.

3.3. Other FGFRs. In contrast to FGFR3 and FGFR1, FGFR2 appears to have a protective or tumour-suppressor role in bladder cancer. Its expression is downregulated in UC and low levels are associated with worse prognosis [70]. Furthermore, FGFR2 re-expression in a UC cell line was associated with reduced proliferation *in vitro* and diminished tumorigenicity in nude mice [71]. No evidence is available regarding FGFR4 and FGFR5 in UC.

3.4. Phenotypic Consequences of Upregulated FGF Signalling in Urothelial Cells. Few studies have investigated the effects of FGFR dysregulation in normal and malignant urothelial cells. Knockdown or inhibition of FGFR3 signalling in the FGFR3-mutant UC cell lines MGHU3 (Y375C), 97-7 (S249C) and UMUC14 (S249C) is accompanied by diminished cell proliferation and/or anchorage dependent growth *in vitro* in all, although with different efficacy [72–75]. Tumorigenic potential *in vivo* is also reduced [72, 74]. Similar effects were seen in UC cell lines RT112 and RT4, which overexpress FGFR3 with no detectable point mutations [74]. These results show that some UC, both FGFR3-mutant and wild-type, have "oncogene addiction" to FGFR3. In contrast, knockdown of FGFR1 in the FGFR1-overexpressing invasive UC cell line UMUC3 did not affect proliferation *in vitro* despite a clear effect on anchorage independent growth and tumorigenicity *in vivo* [68].

Our group has recently begun to elucidate the specific phenotypic differences between FGFR1 and FGFR3 activation in urothelial cells. When mutant FGFR3 was overexpressed in normal urothelial cells, subtle phenotypic changes were observed. The cells had a higher proliferative rate and reduced apoptosis only in confluent cultures, suggesting that activation of FGFR3 signalling may assist premalignant urothelial cells in overcoming cell-cell contact inhibition and favour the formation of hyperplastic bladder lesions [49]. These phenotypes are compatible with the hypothesis that *FGFR3* mutation contributes early in the process of tumour development. FGFR1 overexpression and activation, in contrast, has a more profound effect on proliferation and survival of normal urothelial cells, even in subconfluent culture conditions [68]. As expected, neither mutant FGFR3 or upregulated FGFR1 was sufficient alone to confer on normal urothelial cells a fully transformed phenotype, such as anchorage-independent growth or the ability to form tumours in nude mice [49, 68].

Our recent data, however, show that activation of overexpressed FGFR1 in bladder cancer cell lines is sufficient to induce an epithelial mesenchymal transition (EMT) [76]. EMT developed over a period of 72 hours. Initially a rapid increase in actin stress fibres occurred, followed by an increase in cell size, altered morphology and increased migration and invasion. By using site-directed mutagenesis and small molecule inhibitors, it was shown that combined activation of the mitogen-activated protein kinase (MAPK) and phospholipase C gamma (PLCγ) pathways regulated this EMT. Expression array analysis identified COX-2 as a major upregulated transcript following FGFR1 activation and this led to increased intracellular prostaglandin E_2 levels, which promoted migration. This suggests that the timing and cellular context of FGFR1 dysregulation may be crucial in determining its phenotypic consequences and may influence the development of either superficial or invasive bladder tumours.

Interestingly, despite driving different phenotypes, the signalling pathways activated by FGFR1 and FGFR3 in both normal and malignant cells are similar and involve FRS2, PLCγ1 and ERK1/2 [49, 68]. These observations imply that context-specific downstream effectors of these signalling pathways and interaction with other molecular events need to be elucidated to fully understand the observed phenotypic differences.

4. Clinical Applications

The potential applications of FGFRs in the early diagnosis and clinical management of bladder cancer are summarized in Figure 3.

4.1. Surveillance and Screening. As mutations of *FGFR3* are found in up to 80% of primary Ta tumours, which are characterized by a high recurrence rate, detection of *FGFR3* mutations in urine is currently under study as a noninvasive and inexpensive method for the surveillance of superficial *FGFR3* mutation-positive bladder tumours. A test has been developed to detect eleven common *FGFR3* mutations by multiplex polymerase chain reaction amplification of the three hotspot regions followed by SNaPshot mutation analysis [77]. When applied to urine samples pooled within a 24-hr period, this test is able to detect all mutant tumours irrespective of their size [78]. However, overall sensitivity is around 80%, as it is limited by the fact that around one-fifth of patients with an *FGFR3*-mutant primary tumour have *FGFR3* wild-type recurrences [78, 79].

Detection of *FGFR3* mutations in urine could also be employed for general population screening aimed at early detection of primary tumours. Preliminary results show that a combined test for mutation of *FGFR3, PIK3CA* and *RAS* could potentially detect 75% of primary tumours, including 88% of the pTa-T1G1-2 tumours but only 36% of the high-grade and -stage malignancies [79]. Addition of other markers is being considered to improve detection of invasive tumours [80, 81].

4.2. Prognosis. *FGFR3* mutation status has been investigated as a prognostic marker for recurrence, progression, and survival. A small study including 53 pTaG1-2 tumours showed that wild-type *FGFR3* is predictive of disease recurrence [41]. In contrast, in a subsequent larger study of 764 superficial tumours *FGFR3* mutation was predictive of a higher rate of recurrence in TaG1 but not TaG3 or T1 tumours [28]. In this investigation, TaG2 tumours showed a trend towards a higher recurrence rate but did not reach statistical significance. Whilst an association between *FGFR3* mutation and risk of progression was not detected in this cohort, where progression rate was small [28], other studies suggested a negative correlation [30, 31, 82]. An international prospective study including 221 superficial tumours indicated that *FGFR3* status is not associated with recurrence but is predictive of disease progression in some subgroups (pT1 and high-grade malignancies) [30]. Inverse correlation between FGFR3 mutation and progression in pT1 tumours was also confirmed in a subsequent investigation [83]. A multicentre study comprising 230 superficial tumours suggested that adding *FGFR3* mutation status and Ki-67 positivity to current histopathological criteria improved prediction of progression in about 7% of patients [84]. Furthermore, better survival rates were suggested for patients with muscle invasive tumours harbouring an *FGFR3* mutation [31]. In a recent study, *FGFR3* mutation status was found to be predictive of progression, recurrence, and outcome only when combined with 9p22 LOH status [85], but this was in a relatively small sample set, including only 29 *FGFR3*-mutant tumours. Overall, further research is needed to confirm the utility of *FGFR3*-mutation status as molecular marker for patient stratification alongside current prognostic criteria.

4.3. FGFR-Targeted Therapy in Bladder Cancer. As FGFR3 and FGFR1 are altered in the majority of superficial tumours and in a good proportion of invasive tumours, they represent very inviting therapeutic targets. As discussed in paragraph 3.4, *in vitro* and *in vivo* studies using siRNA or shRNA knockdown or specific antibodies to block FGFRs activity have shown that some UC cell lines are FGFR3-dependent.

A number of FGFR-targeted therapeutic agents have been tested in bladder cancer cell lines *in vitro* and *in vivo*. PD173074 is a selective FGFR-inhibitor, which functions by competing with ATP binding and inhibiting autophosphorylation [86]. TKI258 and SU5402 are broader-profile inhibitors which target both FGFRs and VEGFR [87, 88]. All three compounds were found to be cytotoxic and/or cytostatic on a range of FGFR3- or FGFR1-dependent bladder cancer cell lines *in vitro* and to reduce FGFR phosphorylation and downstream signalling, with PD173074 and TKI258 showing the greatest effect [89, 90]. PD173074 had no toxicity on normal bladder cells, suggesting the existence of a useful therapeutic window, while TKI258 had some detrimental effects, perhaps due to its broader target range [90]. PD173074 was also tested *in vivo* on xenografts obtained from UMUC14, MGHU3, RT112, and SW780 cells and was shown to inhibit growth and induce tumour regression, although growth resumed following drug withdrawal [89, 90]. Notably, response to PD173074 appeared to be related to the level of FGFR expression and dependence, rather than to mutation status. Some *FGFR3*-mutant cell lines (J82, 94-10) were less sensitive than FGFR3-overexpressing cell lines (RT112, RT4, SW780) and other *FGFR3*-mutant cell lines (UMUC14, MGHU3, 97-7). Similarly, in FGFR1-overexpressing cell lines, treatment with PD173074 was effective in JMSU1 but not UMUC3, despite similar levels of FGFR1 expression [68]. This may be attributable to the fact that UMUC3 cells also have a *KRAS2* mutation, which activates the same downstream pathways as FGFR signalling. Alternatively, it is possible that FGFR-dependence may confer an initial survival advantage on bladder cancer cells, which may later be replaced by other oncogenic events. There may therefore be an early "susceptibility window" during which tumours are treatable with FGFR inhibitors as single agents. Overall, the *in vivo* and *in vitro* studies confirm that FGFR inhibitors may be of clinical relevance in the treatment of bladder cancer but also raise some crucial issues, particularly the requirement for biomarkers of FGFR dependence to predict response to treatment and the need for combination therapy with other agents due to the likely recurrence and/or resistance after treatment withdrawal. Clinical studies currently underway with several FGFR inhibitors [91] are hoped to shed light on some of these issues.

FGFR-blocking antibodies represent an alternative approach to the use of small molecule inhibitors. Humanized and fully human synthetic antibodies have recently become available for therapeutic purposes and present several

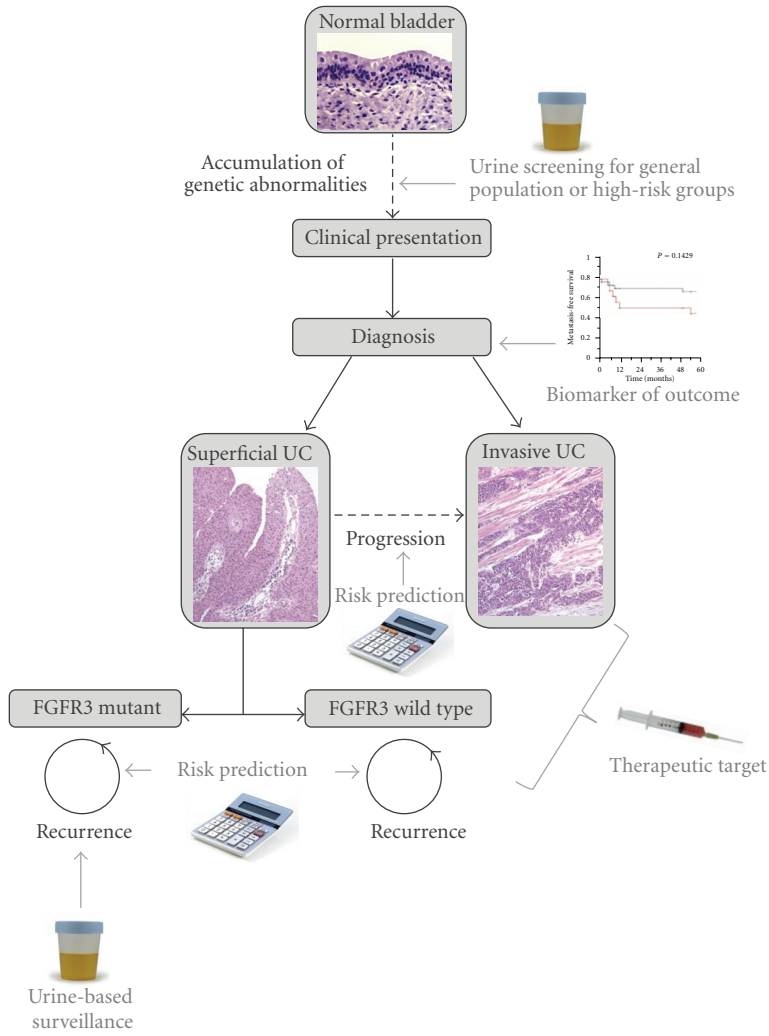

Figure 3: Potential applications of FGFRs in the early detection and clinical management of bladder tumours.

advantages including low toxicity, high target specificity, easy tissue penetration, and the possibility to be combined with immunotoxins or radionucleotides for specific targeting to malignant cells [92]. Results so far are promising. For example, a single-chain Fv against FGFR3 conjugated to the gelonin toxin was shown to block proliferation and induce apoptosis of the FGFR3-overexpressing cell lines RT112 and RT4 both *in vivo* and *in vitro* [93].

The elucidation of downstream targets of FGFR signalling in bladder tumours could also open up new avenues for therapeutic intervention. For example, the discovery that FGFR1 may drive EMT through COX-2 activation [76] suggests that COX-2 inhibition may be particularly beneficial in FGFR1-dependent invasive tumours. A clinical trial utilising a COX-2 inhibitor is currently in progress and it would be interesting to see whether the clinical outcomes correlate with FGFR1 expression and activation levels.

5. Conclusions

In the last decade, it has become clear that FGFRs play a key role in the development of UC and hold promise as therapeutic targets, screening tools, and diagnostic, and prognostic biomarkers. Future challenges include detailed elucidation of downstream signalling, refining FGFR-based screening and prognostic tests, identification of markers to select patients most likely to benefit from FGFR-targeted therapies and development of strategies to overcome recurrence after treatment withdrawal or development of resistance. There is great hope that in the near future the results of research on the role of FGFRs in UC will be translated into the clinical management of these tumours.

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Review Article

Oncolytic Viruses in the Treatment of Bladder Cancer

Kyle G. Potts,[1] Mary M. Hitt,[1] and Ronald B. Moore[1,2]

[1] *Department of Oncology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada T6G 2E1*
[2] *Department of Surgery, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada T6G 2B7*

Correspondence should be addressed to Ronald B. Moore, rbmoore@ualberta.ca

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Bladder carcinoma is the second most common malignancy of the urinary tract. Up to 85% of patients with bladder cancer are diagnosed with a tumor that is limited to the bladder mucosa (Ta, T1, and CIS). These stages are commonly termed as non-muscle-invasive bladder cancer (NMIBC). Although the treatment of NMIBC has greatly improved in recent years, there is a need for additional therapies when patients fail bacillus Calmette-Guérin (BCG) and chemotherapeutic agents. We propose that bladder cancer may be an ideal target for oncolytic viruses engineered to selectively replicate in and lyse tumor cells leaving normal cells unharmed. In support of this hypothesis, here we review current treatment strategies for bladder cancer and their shortcomings, as well as recent advancements in oncolytic viral therapy demonstrating encouraging safety profiles and antitumor activity.

1. Transitional Cell Carcinomas

In the United States, it is estimated that 73,510 men and women (55,600 men and 17,910 women) will be diagnosed with and 14,880 will die of *cancer of the urinary bladder* in 2012, making it the fourth and ninth most common cancers among men and women, respectively [1]. The most common cause for bladder cancer is smoking and other toxin exposure (i.e., petrochemical industry), where the carcinogen is removed from the body by the kidney and stored for long periods of time in the bladder. This results in destabilization of the urothelium resulting in a field effect.

More than 90% of cancers in the bladder are transitional cell carcinomas (TCCs), which have more recently been termed urothelial cell carcinomas [2]. Approximately, 80% of patients with bladder cancer have tumors that are limited to the mucosa of the bladder (stage Ta and carcinoma *in situ* (CIS)) or penetrate into the submucosa (stage T1) [3, 4]. These superficial bladder cancers are now being described as non-muscle-invasive bladder cancer (NMIBC) (Reviewed in [5]). With NMIBC, approximately 70–80% are stage Ta, 20% are T1, and 10% are CIS [6]. Stage Ta tumors are generally low grade, with only about 7% diagnosed as high grade [7]. Stage Ta tumors have a papillary appearance (with increased surface area) and are limited to the urothelium with no infiltration of the deeper lamina propria or underlying muscle. Stage T1 tumors show early invasiveness, crossing the basement membrane into the lamina propria, although not yet invading the deeper muscle layers. There is significant risk of understaging patients with these T1 NMIBCs especially high-grade tumors [8]. CIS (also known as Tis) is restricted to the urothelial layer, but its anaplastic morphology indicates that it is likely a precursor to the development of invasive high-grade bladder cancer. Early, low-grade lesions carry a 50–70% recurrence rate and a 10–15% risk of progression to muscle-invasive disease over a 5-year period [9, 10]. Between 40% and 83% of patients with CIS will develop muscle invasion if left untreated [11, 12]. About 30% of patients with high-grade TCC have muscle-invasive cancer at initial diagnosis, half of whom will go on to have distant metastasis within 2 years, and 60% of whom will not survive 5 years, despite aggressive treatment [8, 13, 14].

2. Treatments for Transitional Cell Carcinoma

Standard therapy combines intravesical therapy with or without transurethral resection (TUR). TUR is typically the first treatment for visible lesions, although this surgery sometimes incompletely removes the tumor, necessitating a second TUR [15, 16]. For patients at low risk of tumor recurrence (and without a bladder wall puncture), early

instillation of a chemotherapeutic agent following TUR is now the standard treatment recommendation. Intravesical chemotherapy, however, is not without risk given that the urothelium is already potentially destabilized by the field effect of carcinogen exposure [17]. Mitomycin C, epirubicin, and doxorubicin have all been determined to be valuable options [18]. High-grade Ta, T1, or CIS tumors put patients at an increased risk for recurrence and, more significantly, progression. Recommended treatment for patients with these high-grade tumors is TUR followed by intravesical treatment with the immunotherapeutic agent Bacillus Calmette-Guérin (BCG) and maintenance immunotherapy for at least 1 year [19, 20].

In patients whose cancer fails to respond to these bladder-sparing treatments and who refuse surgery or are not suitable patients for surgery, the treatment choices become limited. Patients with NMIBC recurrence after intravesical chemotherapy can benefit from BCG instillations [21, 22]. However, if this treatment fails, the treatment options are restricted and comprise a modified immunotherapy treatment, low-dose BCG plus interferon-alpha [23], chemotherapy with intravesical gemcitabine [24, 25] or docetaxel [26]. Cystectomy, however, remains the standard treatment for high-risk patients whose cancers have been unsuccessful with BCG therapy and/or chemotherapy [27]. Patients who receive a cystectomy before their bladder cancer progresses to a muscle invasive disease have shown an excellent disease-free survival [28]. However, cystectomy is not without the possibility of mortality and significant morbidity, especially in the older patient with associated comorbidities [29].

NMIBC that fails BCG is in need of other bladder-sparing treatment options. Here, we will evaluate the potential for the use of oncolytic viruses in the treatment of bladder cancers and try to make a case as to why further clinical evaluation should be pursued.

3. Transitional Cell Carcinoma as a Target for Oncolytic Viruses

Oncolytic virus therapy exploits the altered environment in the tumor cell, allowing the viruses to replicate in and lyse tumor cells, but not normal cells (reviewed in [30–32]). Many different viruses have been examined in preclinical studies for oncolytic properties with several moving into early phase clinical trials. The urinary bladder is an excellent organ to evaluate local oncolytic viral therapy for a number of reasons: (1) the urethra permits easy intravesical instillation allowing the tumor to be exposed to large titers of vector [33]; (2) the bladder is an isolated organ and the trilaminar (asymmetric) unit membrane limits systemic exposure [34–36]; (3) the success of BCG therapy has shown the immunosensitivity of bladder cancer providing a basis for examination of other immunomodulatory agents for therapy [37]; (4) the papillary configuration of NMIBC increases surface area for topical application; (5) there is an urgent need for more bladder-sparing therapies for patients failing conventional therapies.

4. Adenovirus (Ad) as an Oncolytic Agent

Ad is a nonenveloped, linear, double-stranded DNA virus with a genome of approximately 36 kb. The human Ad subgroup C, which contains 2 of the most studied serotypes (types 2 and 5), is widespread in the population and associated with a mild upper respiratory tract infection. Wild-type Ads have been genetically modified to take advantage of the altered tumor environment to allow selective replication. Two general approaches have been used to generate this tumor selectivity. The first is to delete gene functions that are critical for efficient viral replication in normal cells but are expendable in tumor cells [38, 39]. ONYX-015 (dl1520 or CI-1042) was the first conditionally replication-competent engineered Ad to enter a clinical trial. It contains a deletion of the E1B-55 kDa gene and demonstrated oncolytic activity in cancer cells with mutant p53, but only limited cytotoxicity in normal human cells with wild-type p53 function [40, 41] (however, it has become clear that this is not the reason for selective replication) [42]. A second general approach is to limit the expression of the E1A gene product through the use of tumor- and/or tissue-specific promoters [43, 44]. E1A functions to stimulate S phase and transcriptional activation of both cellular and viral genes, allowing virus replication to proceed. An example is the CN706 virus in which the E1A gene is transcriptionally controlled by the PSA promoter, resulting in a virus that selectively replicates in tissue with high PSA levels [45]. There are many other examples of selectively replicating oncolytic Ads that have been reviewed elsewhere [46, 47].

Ramesh et al. have recently reported both preclinical and clinical results of their oncolytic Ad, CG0070 for the treatment of bladder cancer [48]. CG0070 is a selectively replicating Ad in which the human E2F-1 promoter drives expression of the E1A viral gene. E2F-1 is regulated by the retinoblastoma tumor suppressor protein (Rb), which is commonly mutated in many bladder cancers [49–51]. Loss of Rb binding to E2F-1 results in a transcriptionally active E2F [52]. In addition, CG0070 encodes the human granulocyte macrophage-colony stimulating factor (GM-CSF) [53], a cytokine that stimulates the maturation and recruitment of macrophages and dendritic cells and is known to be a potent inducer of local antitumor immunity [54]. CG0070 preferentially replicates in Rb protein-defective bladder cancer cells resulting in production of GM-CSF that activates the host immune response. The tumor selectivity of CG0070 was indicated by the 100-fold higher replication and 1000-fold greater cytotoxicity in bladder TCC cells compared to normal human fibroblast cells. Expression of GM-CSF in MRC-5 (normal lung fibroblast) cells was up to 45-fold lower than in the TCC cell lines used in these experiments. CG0070 showed tumor killing in orthotopic and subcutaneous human xenograft bladder tumor models. A significant antitumor effect was seen after five intratumoral injections of CG0070 at concentrations up to 3×10^{10} viral particles per dose. Half of the mice (5 of 10) treated with the highest dose showed complete tumor regression compared with no regression in mice treated with PBS. GM-CSF expression might enhance the anticancer effect

of CG0070 because uninfected local tumor and potentially distant tumor metastases may be targeted by the induced immune response. However, the human GM-CSF encoded by this virus is species specific; therefore, the antitumor effects seen were likely only a result of the oncolytic activity of CG0070 [55].

These promising preclinical data led to a phase I/II clinical trial with CG0070 that focused on NMIBC (CIS, Ta, and T1 groups) in patients with recurrent bladder cancer after BCG treatment [56]. Results of single and multidose (weekly 6x or monthly 3x) cohorts with CG0070 delivered intravesically into the bladder at doses up to 10^{13} virus particles in 35 patients showed a response rate of 23% in single dose and 64% in multidose groups as assessed by cystoscopy and urine cytology or biopsy. Local toxicities (dysuria, bladder pain, and frequency) and flu-like symptoms were the most common adverse events observed [57, 58]. To our knowledge, this is the first report of a clinical trial using an oncolytic Ad in bladder cancer. The encouraging results have led to a phase II/III trial that is set to begin in mid-2012 evaluating CG0070 in patients with NMIBC who have failed BCG therapy [59].

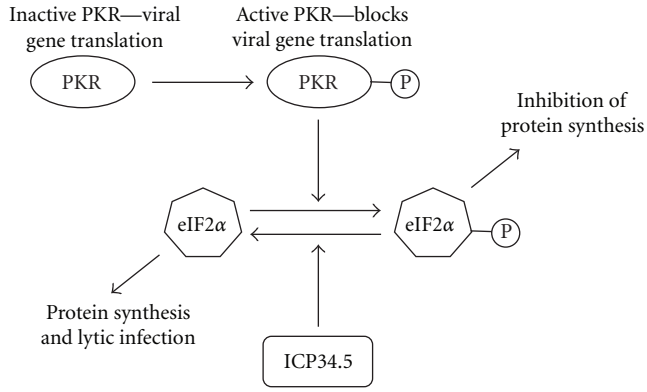

Figure 1: *Oncolytic mechanism of Herpes Simplex Virus.* Viral RNA activates the double-stranded-RNA- (dsRNA-) dependent protein kinase (PKR) by phosphorylation, which causes eIF2α phosphorylation and inhibition of translation of the viral genes. HSV γ-34.5 gene encodes the ICP34.5 protein that acts to dephosphorylate eIF2α allowing protein synthesis to continue. In many cancer cells with activated Ras, PKR is not phosphorylated. Deletion of the γ-34.5 gene from HSV results in attenuation of viral replication in normal cells but allows a lytic infection in cancer cells that have defects in this signaling pathway.

5. Oncolytic Herpes Simplex Virus (HSV)

HSV is a large (150–200 nm diameter) enveloped virus [60] with a double-stranded DNA genome of approximately 150 kb [61]. HSV commonly causes infections in the orofacial region (HSV-1) and in the genital region (HSV-2) (reviewed in [62]). Multiple genetic manipulations to HSV have allowed the development of viruses that selectively replicate in cancer cells. One mutation that has been examined is the inactivation of the viral ICP6 (UL39) gene, which codes for the large subunit of ribonucleotide reductase (RR) [63, 64]. RR plays a key role in making the deoxyribonucleotides (dNTPs) that are needed for DNA synthesis [65]. The RR levels are elevated in dividing tumor cells but low in normal cells. This mutation therefore renders the virus dependent on the cellular enzyme resulting in tumor selectivity. A second modification that has been investigated is the inactivation of the γ-34.5 gene that encodes the ICP34.5 protein which is important for viral replication [66], viral exit from cells [67], prevention of the early shut-off of protein synthesis [68], and neurovirulence [69] (Figure 1). In normal cells, the double-stranded-RNA-(dsRNA-) dependent protein kinase (PKR) shuts off protein synthesis and prevents viral replication [70]. Tumor cells often have defects in this signaling pathway and thus allow viral replication [71]. Mutation of the viral thymidine kinase (UL23) gene also renders the virus dependent on host cell TK expression [72].

Oncolytic HSV armed with immunomodulating transgenes such as GM-CSF [73], interleukin-2 [74], interleukin-12 [75], and B7-1 [76] has also been developed. In addition, conditionally replicating HSV has been used to deliver gene products that convert pro-drugs into cytotoxic agents. One example of this is rRp450, a replication-selective HSV that is deleted for RR and codes for the rat cytochrome *P*450 transgene. Cytochrome *P*450 activates prodrugs such as cyclophosphamide (CPA) to generate highly toxic metabolites. It has been shown *in vitro* that rRp450 oncolytic cell killing was improved by administration of CPA [77]. HSV-1-encoded thymidine kinase (HSV-TK) phosphorylates the prodrug ganciclovir, and the resulting activated metabolite induces increased cell death compared to virus oncolysis alone. HSV-TK activation of ganciclovir in infected cells also stops viral replication [78]. HSV-TK and ganciclovir could therefore be used as a safety mechanism to prevent virus spread if serious virus toxicity were to develop.

Cozzi et al. reported on two attenuated, replication-competent HSVs, G207 and NV1020, for treatment of bladder cancer in a mouse model [79]. Both G207 and NV1020 are genetically modified oncolytic viruses based on HSV type-1 [80, 81]. G207 is modified by deletions of both copies of γ-34.5 and interruption of the UL39 gene (RR) [82]. NV1020 has a deletion in the TK region of the genome and a 15 kb deletion across the junction of the long and short segments of the HSV-1 genome. Both G207 and NV1020 were compared to BCG treatment and proved very successful when delivered by *intravesical instillation* weekly for 3 weeks (10^7 PFU). Ten of 11 animals in the control group revealed bladder tumors at autopsy. A significant increase in tumor clearance was shown in the treated groups, with tumors observed in only six of 12 animals in the BCG group, 5 of 13 animals in the G207 group, and only 2 of 12 animals in the NV1020 group. These encouraging results with oncolytic HSV in bladder cancer suggest that there should be further evaluation of intravesical oncolytic HSV therapies for bladder cancer in clinical trials.

Recently, Simpson et al. have reported results with OncoVEX$^{GALV/CD}$ as an intravesical therapy for bladder cancer. OncoVEX$^{GALV/CD}$ is an oncolytic HSV-1 that expresses a potent prodrug activating gene Fcy::Fur which combines

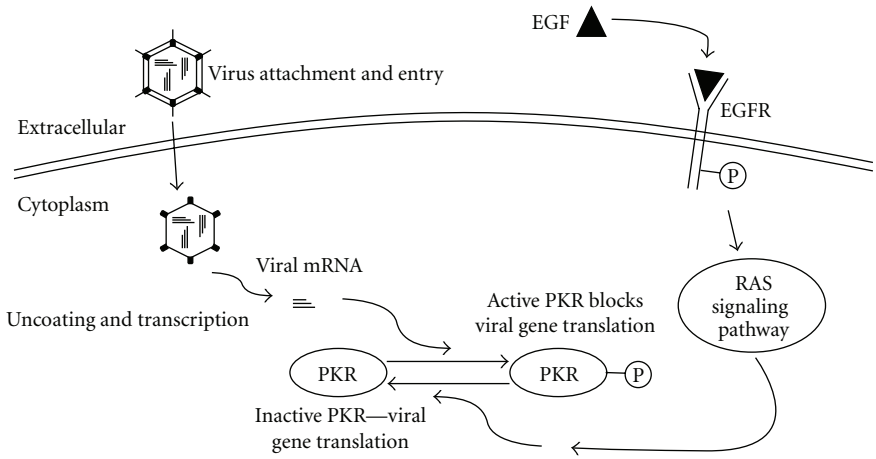

Figure 2: *Oncolytic mechanism of reovirus.* Similar to HSV in normal or untransformed cells, double-stranded reovirus RNA activates the double-stranded-RNA- (dsRNA-) dependent protein kinase (PKR), which causes eIF2α phosphorylation and inhibition of translation of the viral genes. In many cancer cells there is an activated epidermal growth factor receptor (EGFR) or mutation in downstream signaling cascades such as Ras. This dysregulated growth factor signaling means PKR is not phosphorylated thus allowing translation of viral genes and a productive lytic infection that results in cell lysis.

the activity of the yeast cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT) to sensitize cells to 5-fluorocytosine (5-FC) [83]. It also contains the fusogenic gibbon ape leukemia virus envelope (GALV) glycoprotein that can be used to cause an anti-tumor immune response [84]. Deletion of the viral ICP34.5 genes in OncoVEX$^{GALV/CD}$ results in tumor selective viral replication. An 84.5% decrease in tumor size in the presence of both OncoVEX$^{GALV/CD}$ and 5-FC when compared with control was observed in the rat AY27 orthotopic bladder tumor model.

OncoVEX^{GM-CSF} similar in structure to OncoVEX$^{GALV/CD}$ has shown promising results in phase I and II clinical trials for a variety of cancers; including breast, head and neck, and malignant melanoma [85, 86]. It has been modified by deletion of ICP34.5 and replacement of ICP47 with the coding sequence for human GM-CSF under the control of the human cytomegalovirus immediate early promoter [87, 88]. ICP47 blocks the major histocompatibility complex (MHC) class I antigen presentation pathway by binding to the transporter associated with antigen presentation (TAP) protein [89, 90]. As a safety mechanism, the TK gene remains intact, maintaining sensitivity to antiviral agents. A phase II study of OncoVEX^{GM-CSF} in metastatic melanoma demonstrated a 26% objective response rate after direct injection into accessible melanoma lesions. Patients that showed a response had regression of both injected and noninjected lesions [81]. The safety profile of oncolytic HSVs in both the phase I and II studies has been encouraging, and further evaluation is underway with a phase III trial for unresectable stage III or IV melanoma to determine significance [91]. Multiple oncolytic mutants have shown promise in both preclinical bladder cancer models and in clinical trials for other cancers. Thus, there is a huge untapped potential for oncolytic HSV to be used in the treatment of bladder cancer patients.

6. Reovirus

Reoviridae are a family of viruses that includes viruses that infect the gastrointestinal tract and respiratory system. Human reoviruses contain 10 segments of double-stranded RNA and a double shell of proteins that compose the inner capsid or core and the outer capsid.

The first report of the oncolytic properties of these viruses came from the realization that the virus replicated in transformed cell lines but not in normal cells [92]. Since then it has been confirmed that reovirus oncolysis requires overexpression of the Ras-signaling cascade in target cells or upregulated growth factor signaling [93, 94]. In normal cells, reovirus (double-stranded RNA) activates the double-stranded RNA-dependent protein kinase (PKR) and blocks viral protein translation by inhibiting the eukaryotic initiation factor 2α (eIF2α) [95]. In cancer cells with activated Ras, reovirus-activated protein kinase activation is inhibited, allowing viral protein synthesis and an oncolytic infection to occur (Figure 2). Around 30% of all cancers have a mutation in the Ras protein [96]. The majority of the remaining cancers still rely on some form of mutation in the epidermal growth factor (EGF) pathway. This can occur through mutation of other downstream elements or from growth factor ligand/receptor interactions that initiate Ras function. Mutated receptor tyrosine kinase proteins that are constitutively active can also occur [97]. Up to 90% of TCC have an overactive EGF pathway [98].

Hanel et al. demonstrated oncolytic activity of reovirus *in vitro* and in an orthotopic bladder tumor model [99]. Female rats were treated twice a week for 3 weeks with low, medium, and high doses (2.5×10^5, 2.5×10^6, 2.5×10^7 PFU) of intravesical reovirus or BCG as control. Complete tumor response was observed in 90% at 100 days after tumor implantation in medium- and high-dose reovirus-instilled animals, while the highest survival in the BCG-treated

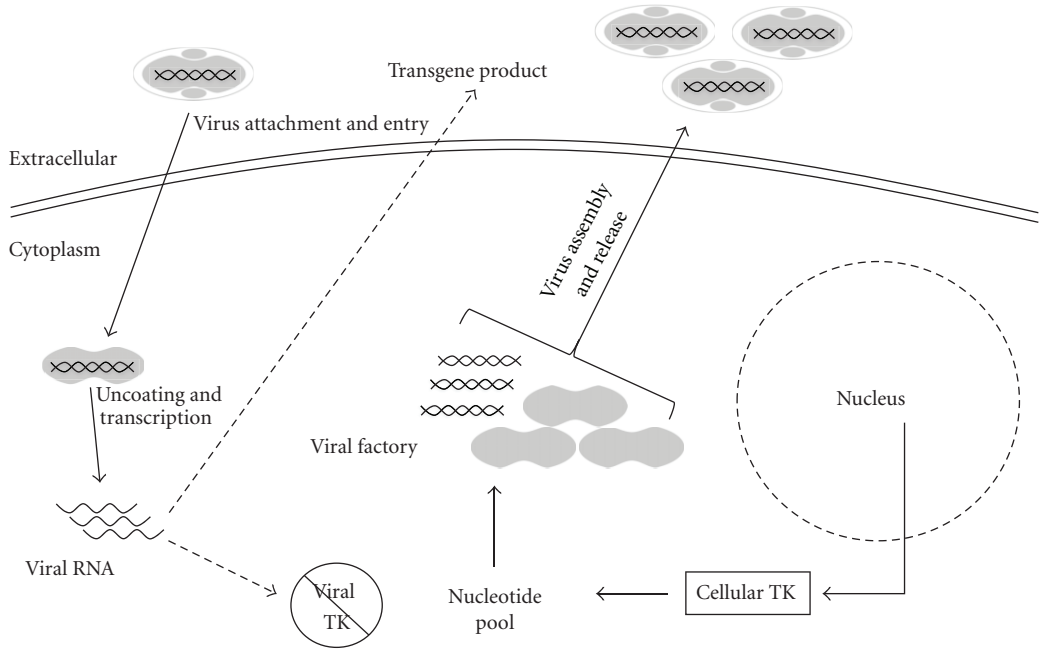

Figure 3: *Tumor selectivity of oncolytic vaccinia viruses.* In normal cells, the wild-type virus encodes a range of gene products that allow virus replication in the cytoplasm of host cells. These products include, but are not limited to, thymidine kinase (TK) and ribonucleotide reductase (not shown), which generate a nucleotide pool to facilitate virus replication. In normal cells, viruses deleted of these essential genes are unable to undergo productive replication. However, in tumor cells, mutations cause dysregulation of numerous pathways, including pathways that allow for unchecked proliferation. One result of these unregulated proliferative pathways is a high level of production of nucleotides, creating a favorable environment for vaccinia virus replication. The mutated viruses are able to replicate, express transgenes (if present), and lyse tumor cells.

groups was 50%. Despite these encouraging results, little research has gone into further use of reovirus for bladder cancer.

In the first-in-man study, patients with a variety of malignancies received escalating doses of intratumoral reovirus at levels ranging from a single injection of 10^7 PFU to three injections of 10^{10} PFU. The main symptoms were headaches and a flu-like illness [100]. Since then, multiple phase I and II studies have been completed. A phase I dose escalation study was performed on 12 patients with recurrent gliomas, evaluating an unmodified reovirus administered through intratumoral injection. A maximum tolerated dose was not reached, and treatment was well tolerated [101]. A phase II study was also performed with i.v. administration of wild-type reovirus in patients with bone and soft tissue sarcomas that had metastasized to the lung [102]. These clinical studies show that both intratumoral and i.v. administration of wild-type reovirus in patients was safe and well tolerated. These early clinical results, as well as the relatively low risk due to reovirus' limited pathogenicity in humans, highlight the promising potential for this oncolytic agent to expand its clinical potential to include bladder cancer.

7. Oncolytic Vaccinia Virus (VAC)

VAC has a large (~200 kb) linear double-stranded DNA genome that replicates exclusively in the cytoplasm. VAC infects many different cell types with high efficiency. VAC encodes many of the proteins required for robust virus replication in normal cells (reviewed in [103–105]).

In recent years, there has been extensive research into VAC as a cancer therapy. Genetic mutations that occur in cancer can generate an environment that is optimal for VAC replication; thus, some of the viral genes involved in replication become expendable. Therefore, deletion of these genes from the viral genome greatly reduces the ability of the virus to replicate productively in most normal cells, while allowing them to retain their replication ability in cancer cells. A range of VAC gene deletions with such properties has been investigated as a means to increase tumor selectivity of the virus. Oncolytic VACs reported to date are most commonly generated by mutations that inactivate J2R (thymidine kinase, TK) and C11L/R (vaccinia growth factor, VGF), which reduce virulence in the host (animals) and favors virus growth in rapidly dividing cells [106, 107]. Cellular TK is briefly expressed during S phase in normal cells but is constitutively expressed at high levels in a large number of cancers throughout the cell cycle (Figure 3) [108]. VGF is an EGF homologue that can bind to cellular EGF receptor [109, 110]. VGF is released from infected cells to induce proliferation, and VAC strains with VGF deletions show selective replication in cancers with an activated EGFR. The VGF deletion can be combined with the TK deletion to generate a further attenuated virus [111]. Recently, Gammon et al. have shown that, by deleting the gene encoding the small subunit of VAC RR (F4L), one can

render the virus highly dependent upon the cellular homolog to provide the complementing activity that is needed for virus replication [112]. The F4L deleted viruses are thus quite highly attenuated in infected animals and show a tropism that greatly favors cells containing high levels of RR. This virus may be specifically useful for treating recurrent NMIBC where patients have failed BCG and RR-targeted chemotherapies such as gemcitabine.

Oncolytic VACs armed with a variety of transgenes have also generated much attention recently. Viruses have been developed that encode cytokines such as GM-CSF [113] and interferon-beta (IFN-β) [114]. Interestingly, VAC encodes an inhibitor of type-I IFNs, the B18R gene product. An oncolytic VAC has been constructed with deletion of the B18R gene and insertion of the INF-β gene. VAC replication should be highly restricted in normal cells, but permissive in IFN-resistant cancer cells. Furthermore, IFN-β is predicted to elicit an increased anti-cancer response [115]. Anti-angiogenic agents have been expressed to help complement the oncolytic effects of the virus [116]. Finally, prodrug-converting enzymes have been introduced into VACs to convert nontoxic prodrugs into toxic products within the tumor [117].

Gomella et al. reported a phase I study where increasing doses of intravesical wild-type VAC (the Dryvax vaccine) were administered to patients with muscle-invasive-bladder carcinoma for whom radical cystectomy was planned as final treatment [118]. The study examined 4 patients that were treated 3 times over 2 weeks with a maximum dose of 10^8 PFU prior to cystectomy. This study demonstrated that even wild-type VAC can be administered safely into the bladder and cause the recruitment of lymphocytes and induction of a local inflammatory response. Besides mild local toxicity, no serious treatment-related side effects were reported. The excellent patient tolerance of intravesical VAC and the significant immune infiltrates seen after instillation support the potential use of VAC as an oncolytic agent for intravesical bladder cancer therapy.

Clinical data have now been published for the first targeted and armed oncolytic poxvirus to be used in the clinic, JX-594. It is a Wyeth strain VAC with inactivation of J2R (viral TK) and insertion of the GM-CSF gene [119]. Phase I clinical data reported on the intratumoral injection of seven patients with surgically incurable cutaneous melanoma [120]. Multiple injections with JX-594 at doses up to 2×10^7 PFU/lesion were given over 6 weeks. Overall the treatment had controlled side effects that included transient flu-like symptoms and local inflammation, with the occasional pustule formation at the site of injection. Five of seven patients had some response to the treatment with one patient having a complete remission. In another phase I trial, direct injection of JX-594 into liver tumors was well tolerated, with virus replication, expression of active GM-CSF, and tumor killing observed [121]. In this dose escalation study, patients who had previously received multiple therapies were injected with up to 3×10^9 PFU every 3 weeks with an average of 3.4 treatments. Of the ten patients assessed, three showed partial responses, six had stable disease, and one showed progression. JX-594 was generally well tolerated up to the maximum tolerated dose of 10^9 PFU. The dose-limiting toxicity, hyperbilirubinemia, was seen at 3×10^9 PFU because of tumor swelling, causing a bile-duct obstruction.

Partial results of a phase II trial examining intratumoral administration JX-594 in patients with hepatocellular carcinoma have been reported. They reported that 6-month survival of patients treated with low-dose (10^8 PFU) was 48%, and with high-dose (10^9 PFU) was 75%. The 12-month survival was 18% and 75%, respectively [122]. Efficient tumor killing seems to be a dose-dependent property that can be limited by toxicity following systemic or hepatic delivery. JX-594 has recently been tested in a phase I dose-escalation trial through i.v. administration in 23 cancer patients with advanced solid tumors that had developed resistance to multiple other treatments. This study established a maximum feasible dose of 3×10^7 PFU/Kg (equivalent to a total dose of about 2×10^9 PFU) [123]. This is the first report of replication and transgene expression in metastatic tumors after i.v. administration of an oncolytic virus. Because of the anatomical isolation of the bladder, it may be possible to administer higher doses of virus locally without systemic effects. Encouraging clinical results for the treatment of other cancers with oncolytic VAC further suggest that the investigation of oncolytic VAC as a bladder cancer therapy should be a priority.

8. Conclusion

Surgery, BCG, and chemotherapy dramatically slow the progress of bladder cancer but do not eradicate the disease totally. Patients with NMIBC that fail BCG therapy are in need of other bladder-sparing treatment options. This paper discussed the potential of oncolytic viruses as a treatment option in bladder cancer. Encouraging safety profiles and antitumor activity have been demonstrated with a variety of oncolytic viruses. However, very little preclinical, let alone clinical, data have been reported for oncolytic viruses in bladder cancer.

Although the agents described in this paper have shown convincing preclinical and early clinical results, the ultimate proof of antitumor efficacy and safety still need to be provided by randomized phase III clinical trials. Therefore, there remains uncertainty in their ability to have significantly better effects over current therapies in patients. As with any viral therapy, the main obstacle to overcome is delivery of sufficient virus particles to the target tissue in order to have a desired therapeutic effect. However, the bladder may provide an environment to help overcome some of these issues. Local delivery to the isolated environment of the bladder can allow the tumor to be exposed to large titers of virus with limited systemic exposure and consequent toxicity. The papillary configuration of the NMIBC lends itself to topical application of agents with tropism to urothelial cancer. Furthermore, these agents appear to be noncarcinogenic like BCG and but unlike BCG could potentially be administered earlier in the course of therapy (immediately after TUR) without the significant risk of severe systemic illness. In

addition, direct oncolysis by selective replication in transformed NMIBC cells could potentially avoid inflammation and the profound symptoms of cystitis. Combinations of these viral agents targeting multiple or sequential pathways could prevent the development of resistance, with little added toxicity. Thus, the potential high degree of safety and efficacy predicted for oncolytic virus therapy of urothelial cancer warrants immediate further investigation at both the preclinical and clinical levels.

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Review Article

Epigenetic Alterations in Bladder Cancer and Their Potential Clinical Implications

Han Han,[1,2] Erika M. Wolff,[2] and Gangning Liang[2]

[1] *Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90033, USA*
[2] *Department of Urology, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA*

Correspondence should be addressed to Gangning Liang, gliang@usc.edu

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Urothelial carcinoma (UC), the most common type of bladder cancer, is one of the most expensive malignancies to treat due to its high rate of recurrence. The characterization of the genetic alterations associated with UC has revealed the presence of two mutually exclusive molecular pathways along which distinct genetic abnormalities contribute to the formation of invasive and noninvasive tumors. Here, we focus on the epigenetic alterations found in UC, including the presence of an epigenetic field defect throughout bladders with tumors. A distinct hypomethylation pattern was found in noninvasive tumors, whereas widespread hypermethylation was found in invasive tumors, indicating the two pathways given rise to two tumor types also differ epigenetically. Since certain epigenetic alterations precede histopathological changes, they can serve as excellent markers for the development of diagnostic, prognostic, and surveillance tools. In addition, their dynamic nature and reversibility with pharmacological interventions open new and exciting avenues for therapies. The epigenetic abnormalities associated with UC would make it an excellent target for epigenetic therapy, which is currently approved for the treatment of a few hematological malignancies. Future research is needed to address efficacy and potential toxicity issues before it can be implemented as a therapeutic strategy for solid tumors.

1. Introduction

Bladder cancer is one of the most commonly diagnosed malignancies in the United States, with an estimated number of 73,510 new cases and 14,880 deaths in 2012 [1]. Worldwide, bladder cancer is the seventh most common malignancy [2]. The risk factors associated with development of bladder cancer include cigarette-smoking, exposure to chemicals, such as aromatic amines, chronic bladder inflammation, genetic predisposition, and age [3, 4]. In the United States, more than 90% of bladder tumors are diagnosed as urothelial carcinoma (UC), 5% as squamous-cell carcinoma (SCC), and 2% as adenocarcinomas [5]. In countries, where chronic urinary infection by *Schistosoma haematobium* is prevalent, most bladder cancers are SCC [6]. Due to the low incidence of SCC in the US as well as the rest of the Western countries, this paper primarily focuses on UC. Of all newly diagnosed UC cases, approximately 80% are noninvasive papillary tumors, which are confined to the urothelium (CIS, Ta) or lamina propria (T1). The remaining 20% of tumors are muscle invasive (T2–T4) and are typically treated by radical cystectomy [7]. Despite the fact that most noninvasive UCs can be successfully treated by transurethral resection of bladder tumor (TURBT), 70% of patients will suffer tumor recurrence after the initial treatment and 10–20% of those recurrent tumors will become invasive. Specific genetic alterations characterize UCs; for instance, noninvasive tumors show frequent mutations in fibroblast growth factor receptor 3 (FGFR3) mutations; whereas invasive tumors often display *TP53* mutations. Further progression of noninvasive tumors to invasive tumors requires subsequent mutations in *TP53* (Figure 1) [4, 8]. The high rate of recurrence and inability to predict which tumor will progress require frequent and invasive clinical management after the initial treatment.

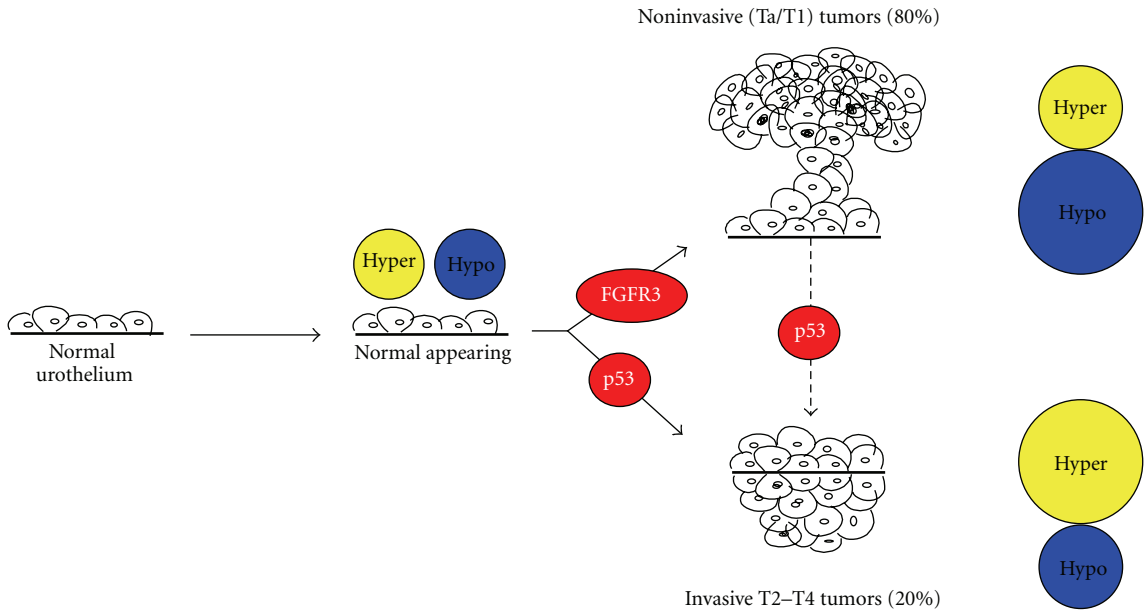

Figure 1: Two distinct molecular pathways for the initiation and progression of urothelial carcinoma. Normal urothelium acquire both aberrant DNA hypermethylation and hypomethylation, prior to the onset of genetic mutations. Normal-appearing urothelium then can transform into either noninvasive (Ta/T1) tumors or invasive tumors (T2–T4) through the accumulation of activating mutations of FGFR3 (fibroblast growth factor receptor 3) or *TP53*, respectively. Approximately, 80% of all newly diagnosed cases are noninvasive papillary tumors, which do not often progress to invasive tumors. Acquiring subsequently *TP53* mutation is necessary for the progression. Noninvasive tumors acquire less hypermethylation and more aberrant hypomethylation, among which a group of genes is distinctively hypomethylated in noninvasive tumors. Invasive tumors display the reversed methylation profile.

Currently, the gold standard for bladder cancer diagnosis and surveillance is cystoscopy, which is an invasive and expensive method that allows direct visualization of the bladder. Noninvasive methods are also available, but the majority of them lack sensitivity. Urinary cytology is the most widely used noninvasive method for detecting the presence of cancerous cells in urine and is often used in conjunction with cystoscopy. However, this method shows poor performance in detecting low-grade tumors [5, 9]. Furthermore, the accuracy of urinary cytology is jeopardized by interobserver variability [5]. The current recommended post-TURBT surveillance regimen for tumor recurrence involves a combination of cystoscopy and voided urine cytology every three months for two years and once a year thereafter [10]. This results in $2.2 billion annual expenditure, making bladder cancer one of the most expensive malignancies to treat [4, 11, 12]. In recent years, much effort has been dedicated to the discovery of tumor biomarkers that represent tumor properties to overcome the limitations of cystoscopy and cytology. Although some progress has been made in this area with some biomarkers showing considerable clinical values, the majority of them lack sensitivity and/or specificity [13]. To date, no biomarker assay stands alone to detect and monitor the disease. Therefore, the elucidation of the molecular mechanisms that underlie the high rate of recurrence shown by bladder tumors will help to develop more accurate and cost-effective noninvasive strategies for diagnosis, prognosis, and surveillance of the disease.

2. Genetic Mutations Associated with Invasive and Noninvasive Urothelial Carcinoma

Many types of invasive carcinomas, including colon cancer [14], arise from noninvasive carcinomas via the accumulation of mutations over time. However, pioneering work done by our group has demonstrated that such a developmental continuum does not exist in UC. There is substantial evidence for the existence of two mutually exclusive molecular pathways that lead to bladder carcinogenesis in which distinct genetic alterations are responsible for the formation of noninvasive and invasive tumors, resulting in divergent clinical behaviors [15]. Noninvasive tumors usually arise by tissue hyperplasia and show mutations in fibroblast growth factor receptor 3 (FGFR3) [16, 17], which is involved in cell differentiation and angiogenesis [18]. Patients with such tumors usually do not show disease progression, but experience frequent recurrence [8, 19]. Invasive tumors are believed to arise by tissue dysplasia and often harbor mutations in *TP53* [15, 20], a critical tumor suppressor gene that initiates cell-cycle arrest upon DNA damage [21]. These tumors are aggressive and associated with high mortality [8]. These two pathways do not occur sequentially and only under rare circumstances, when a subsequent p53 mutation is acquired, noninvasive tumors can progress to invasive tumors [15]. The genetic alterations associated with UC are relatively well defined as compared to its epigenetic alterations. Therefore, this paper mainly focuses on the epigenetic aberrations found in UC.

3. The Epigenetic Landscape and Its Deregulation in Urothelial Carcinomas

Epigenetics encompasses the heritable changes in gene expression that are not caused by changes in the underlying DNA sequence [22]. Such epigenetic changes include DNA methylation, histone modifications, and nucleosome positioning [15, 22–24]. Among the three layers of epigenetic regulation, DNA methylation was the first to be identified and is the most extensively studied. It involves the covalent addition of a methyl group to the 5′ position of cytosine residues in the context of CpG dinucleotides. The distribution of CpG sites is asymmetrical and nonrandom throughout the genome, with a high frequency of CpG sites occurring near promoters (CpG islands) and repetitive elements [25, 26]. The majority of promoter-associated CpG islands are usually not methylated under normal conditions, with the exception of imprinted genes [25, 27]. DNA methylation at gene promoters modifies DNA accessibility to transcription factors or helps recruit silencing-associated proteins, resulting in gene silencing [28, 29].

The N-termini of histones undergo a variety of posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitination, and sumoylation to generate transcriptionally permissive or refractory chromatin conformations depending on the type and location of the modification [23, 30]. For instance, trimethylation of lysine 4 on Histone 3 (H3K4me3) is enriched at the promoters of transcriptionally active genes [31], whereas trimethylation of H3K9 and H3K27 is associated with transcriptionally inactive gene promoters [23]. The balanced activity of histone modifying enzymes that add or remove specific modifications is critical for normal cell physiology [32]. In addition, the presence of specific histone variants at regulatory regions also plays a role in controlling gene expression by influencing the stability of nucleosome occupancy [33], which either facilitates or prevents binding of transcription machinery at transcription start sites [34, 35].

In addition to genetic abnormalities, epigenetic alterations also play vital roles in the initiation as well as progression of cancer. Global disruption of the epigenetic landscape, resulting in aberrant gene expression and function, is a hallmark of human cancer [27, 36]. The cancer methylome is highly disrupted, featuring hypermethylation and aberrant silencing of tumor suppressor genes, and hypomethylation of repetitive sequences, transposons, and oncogenes, which contributes to tumorigenesis by increasing chromosomal instability and activating aberrant transcripts [36–38]. Substantial evidence shows that the epigenome of UC cells displays profound alterations in DNA methylation, histone modifications, and nucleosome positioning. In this context, a few well-known tumor suppressor genes, including *CDH1*, *CDH13*, *INK4A*, *RASSF1A*, *APC*, *ARF*, *MLH1*, and *DAPK* [39–41], have been reported to be frequently hypermethylated and silenced in UC, resulting in deregulated cell proliferation [42]. In addition to global hypomethylation of repetitive elements, such as long interspersed nuclear elements (LINE-1) [43], work done by our group has demonstrated that a specific LINE-1 located within the mesenchymal-epithelial transition factor (MET) oncogene (L1-MET) is hypomethylated and transcriptionally active in UC, accompanied by the presence of a nucleosome-depleted region (NDR) just upstream of the transcription start site (TSS), active histone marks, and the histone variant H2A.Z [44]. Recent advances in high-throughput technologies have facilitated the identification of distinct DNA methylation, gene expression, and histone modification profiles associated with tumors, including UC [45–48]. Such technologies will aid in establishing a comprehensive understanding of the altered epigenome present in the diseased state, and subsequently facilitate the identification of potential drug targets and biomarkers for diagnostic and prognostic purposes.

The two mutually exclusive molecular pathways for the formation of noninvasive and invasive tumors also differ epigenetically in addition to genetically. A genome-wide analysis of DNA methylation patterns in noninvasive and invasive urothelial tumors revealed a distinct hypomethylation pattern only in noninvasive tumors and widespread hypermethylation in invasive tumors, suggesting that they arise via distinct epigenetic pathways [46]. When correlations between DNA methylation and gene expression were performed, an inverse relationship was observed for most genes, highlighting the functional significance of both aberrant DNA hypermethylation and hypomethylation of gene promoters in tumors. Many of the hypomethylated loci distinctively associated with noninvasive tumors are non-CpG island promoters of tissue-specific genes. The unique hypomethylation pattern present in the noninvasive tumors may explain the failure of these tumors to become invasive [46].

4. Epigenetic Field Defect

The alarmingly high recurrence rate of bladder cancer is of clinical concern, highlighting the need for physicians and scientists to elucidate its underlying mechanism. The presence of a field defect, an area of tissue that is predisposed to undergo oncogenic transformation, has been postulated to be responsible for such high recurrence rate [49]. This concept was first introduced by Slaughter et al., who found abnormal tissues composed of epithelial cells of polyclonal origins surrounding oral squamous cell carcinomas [50]. Since then a field defect, as identified by genetic alterations, has been found in tumors arising from various tissues, including upper aerodigestive tract [51], lung [52], esophagus [53], vulva [54], cervix [55], colon [56], skin [57], and bladder [58, 59].

In addition to genetic field defects, epigenetic field defects have also been found in various types of cancer, including stomach [60, 61], liver [62], colon [63–65], lung [66], breast [67], kidney [68], and esophageal [69]. Using the Illumina GoldenGate assay to compare primary tumors, normal-appearing tissues at 0.5 cm increments away from the tumor in multiple directions, and urothelium from cancer-free bladders, our group found that cancer-bearing bladders have a widespread epigenetic field defect [46]. Methylation at a significant number of loci (169 probes spanning 155

unique gene regions) was altered not only in tumors but also in normal-appearing urothelial taken at least 5 cm away from the corresponding primary tumor, with the majority of the loci, such as *ZO2*, *MYOD1*, and *CDH13,* being aberrantly hypermethylated [46]. Among the 169 loci, 145 loci displayed a trend of increasing methylation in invasive tumors and 41 loci in noninvasive tumors, indicating that hypermethylation may constitute the majority of epigenetic defects present in the urothelium. In addition, we also observed hypomethylation and ectopic expression of L1-MET in primary tumors and surrounding histologically normal tissues [44]. Together, these studies suggest that uniquely hypermethylated or hypomethylated loci that are found in bladder tumors and surrounding tissues may serve as biomarkers and could be used to develop diagnostic, prognostic, and/or surveillance tools.

The field defect found in tumor-bearing bladders could be propagated by clonal expansion or a generalized epigenetic field defect. Clonal expansion involves the process of accumulating aberrant DNA methylation in one cell, followed by expansion of that cell population across the urothelium, resulting in subsequent transformation. Analysis of the pattern of X-chromosome inactivation, which is maintained during clonal expansion, in samples taken from 2 female patients indicated that the widespread epigenetic field defect observed in UC could not be attributed to clonal expansion. Instead, it is likely that epigenetic alterations occur independently in many cells across the urothelium, thereby predisposing them to undergo oncogenic transformation [46]. The urothelium is uniformly exposed to carcinogens, causing epigenetic alterations, initially without associated histological changes. It is plausible that at the initiation of UC, there is no "normal" urothelium present and this may provide an explanation for its high recurrence rate after TURBT. The altered epigenome in the normal-appearing urothelium may allow for a more permissive environment for the growth of newly transformed cells.

5. Using DNA Methylation as a Marker for Diagnosis, Prognosis, and Surveillance

Since bladder cancer may remain asymptomatic until a relatively late stage, ideal clinical management would be comprised of early detection, accurate prediction of disease progression, and frequent monitoring. However, unlike many other types of cancers, there is no standard and effective noninvasive strategy for early detection [70]. Currently, conventional histopathological evaluations that are used for the categorization of tumor grade and stage are also used to predict the potential behavior of tumors. Such histopathological evaluations are not accurate in predicting the behaviors of heterogeneous tumors, resulting in significant differences in clinical outcomes for patients with tumors of similar stages [71]. Therefore, patients undergo frequent and long-term surveillance after the initial treatment. There is a strong need to develop economically viable, noninvasive methods with high sensitivity and specificity for diagnosis, prognosis, and monitoring of UC. A better understanding from both a genetic and an epigenetic perspective of how UC arises and progresses has greatly contributed to the ongoing efforts to create these new assays.

The ability to detect cancer-specific genetic and epigenetic alterations in cells detached from the urothelium, which can be found in voided urine samples, supports the use of such biomarkers in the development of noninvasive methods for bladder cancer detection and progression [40, 72–75]. Several of the most promising genetic biomarkers whose protein or expression levels are upregulated in the diseased state, including nuclear matrix protein 22 (NMP-22), telomerase, and the nuclear matrix protein bladder cancer 4 (BLCA-4), have been reported to have promising values [73]. However, they suffer from similar limitations as urine cytology—low sensitivity for low grade tumors. Although some of the markers have been used to complement cystoscopy and urinary cytology, none of them has been utilized independently [73]. The detection of genetic mutations DNA extracted from urine sediment is another screening method, and mutations of the fibroblast growth factor 3 (FGFR3) gene, which frequently occur in superficial bladder tumors, can be readily identified by this method, providing greater sensitivity in the detection of TA tumors than cytology [76].

A greater understanding of the roles epigenetics plays in tumorigenesis has opened up new avenues for developing innovative diagnostic and prognostic biomarkers. Because of their early onset in bladder tumorigenesis and presence in precancerous lesions and tissues surrounding primary tumors (field defect), DNA methylation changes are excellent biomarker candidates [46, 74]. Tumor-associated alterations in DNA methylation are readily detectable in body fluids, such as blood [77] and urine [72, 78]. We have shown that DNA isolated from urine and primary tumors of bladder cancer patients show similar methylation profiles, displaying hypermethylation at a number of apoptosis-associated genes, including *DAPK*, *BCL2,* and *TERT*. These loci are not methylated in urine specimens from healthy controls [72], suggesting that such tumor-specific methylation markers have the potential to serve as diagnostic tools using a noninvasive sample procurement method. Numerous studies have identified a number of additional methylation marks suited for urine-based detection, including the combination of TWIST and NID2 [79] and the combination of E-cadherin, p14, and RASSF1A [80]. Costa and collaborators reported 100% sensitivity and 94% of specificity for early stage Ta and low-grade UC when evaluating DNA methylation changes in a panel of 3 genes: *GDF15*, *TMEFF2,* and *VIM* [74]. Reinert and collaborators established a detailed mapping of the methylome in bladder cancer and identified four novel DNA methylation marks: *HOXA9*, *ZNF154*, *POU4F2,* and *EOMES* [75]. It is of interest that the methylation status of genes that show nontumor specific DNA methylation patterns can be potentially used for assessing prognosis and risk for recurrence. This category includes genes that are aberrantly methylated in histologically normal tissues surrounding bladder primary tumors, such as *ZO2*, *MYOD1,*and *CDH13* [46].

The technological advances in the detection of global methylation patterns have facilitated the characterization of tumor methylomes, thereby providing new opportunities to find better and more sensitive biomarkers. Although efforts in this regard are currently underway, more studies are needed to translate these findings into the clinical setting.

6. Urothelial Carcinoma and Epigenetic Therapies

Although epigenetic modifications are heritable, their dynamic nature and reversibility through pharmacological interventions make them excellent targets for anticancer therapies. Over the past few decades, various drugs aimed at targeting different types of epigenetic alterations observed in cancer, including DNA methylation and histone modifications, have been developed, with the goal of reactivating aberrantly silenced genes. In addition to having genetic abnormalities, UC is also driven by progressive alterations in the epigenome, resulting in changes in chromatin packaging and aberrant gene expression [46]. Epigenetic changes in UC have been well elucidated and their significance has been demonstrated, making UC a suitable candidate for epigenetic therapy. Due to the presence of an epigenetic field defect in UC, epigenetic therapies may also prevent recurrence by reversing the epigenetic aberrations occurring in histological normal tissues that remain after TURBT.

UC is an excellent candidate for epigenetic therapy due to the presence of a highly disturbed epigenome, which can be restored via the intervention of epigenetic agents. Promoter hypermethylation accompanied by histone modifications which facilitate the formation of heterochromatin is commonly seen in UC. DNA methyltransferase inhibitors (DNMTi) and/or histone deacetylase inhibitors (HDACi) could be used to reverse such abnormalities and restore the expression of aberrantly silenced genes. In addition to having therapeutic value, epigenetic therapies also have preventive value in patients who had undergone TURBT, which leaves large areas of epigenetically altered tissues. Our lab has demonstrated that *ZO2*, which is methylated in tumors and adjacent normal-appearing tissues, is reactivated upon 5-Aza-2-deoxcytidine (5-Aza-CdR) treatment in a panel of bladder cancer cell lines [46]. Treatment with DNMTi also has the potential to reverse the invasiveness of high-grade tumors by creating an epigenetic profile similar to that of low-grade tumors. As discussed above, noninvasive tumors show a unique hypomethylation pattern in the vicinity of TSSs which may account for their failure to acquire an invasive phenotype.

7. DNA Methyltransferase Inhibitors

The widespread hypermethylation at promoters in UC, particularly in invasive tumors [40, 41, 46] suggests that restoration of a normal epigenome through the use of DNA hypomethylating agents would be clinically beneficial. Many of these agents are nucleoside analogues, which get incorporated into DNA and sequester DNA methyltransferases (DNMTs), resulting in depletion of DNMTs and global hypomethylation upon subsequent cell divisions [81].

Two DNA methylation inhibitors, 5-Azacytidine (5-Aza-CR; Vidaza) and 5-Aza-2-deoxycytidine (5-Aza-CdR; Decitabine), have been approved by the Food and Drug Administration (FDA) for the treatment of myeloid malignancies [81]. Both are cytosine analogues that are incorporated into replicating DNA in the place of cytosine, resulting in heritable global demethylation [32, 82]. In addition, 5-Aza-CR is also incorporated into RNA, which prevents the translation of oncogenic proteins [83, 84].

Despite their promising results in treating myeloid malignancies, both 5-Aza-CdR and 5-Aza-CR have limited efficacy in treating solid tumors due to their plasma instability, cytotoxicity, and potentially mutagenic properties [85–87]. The instability of 5-Aza-CR and 5-Aza-CdR is attributed to hydrolysis and deamination, presenting a challenge for their clinical application. To address this issue, several cytidine analogues with improved stability and efficacy have been developed. Zebularine, which lacks an amino group in the 4-position of the pyrimidine ring, is less chemically labile and cytotoxic than the 5-Aza analogs. Studies have shown that it reactivates aberrantly silenced tumor suppressor genes in breast cancer cell lines [88] and inhibits polyp formation in female *MIN* mice [89]. Another method used to increase drug stability is to generate them as prodrugs. An example of this type of analogue is S110, a dinucleotide containing the 5-azacytosine ring that is less prone to deamination and less cytotoxic. S110 has been shown to induce *p16* expression by reducing DNA methylation in human xenografts [90].

In the past few years, tremendous efforts have been invested into broadening the application of 5-Aza-CdR and 5-Aza-CR to the treatment of solid tumors. A preclinical phase I trial in which 5-Aza-CR was subcutaneously administered to 19 dogs with naturally occurring invasive UC showed favorable tumor response. 72% of the dogs have demonstrated either partial remission or stable disease, meriting potential application of such treatment in humans [91].

8. Histone Deacetylase Inhibitors

Another layer of epigenetic regulation includes posttranslational modifications of histones, which play an important role in gene expression by altering chromatin structure [92]. The type and location of histone modifications determine the conformation of chromatin. Certain modifications, such as H3K4me3 and H3K9 acetylation, are associated with euchromatin and make the DNA more accessible to the transcriptional machinery. Other modifications, such as H3K9me3 and H3K27me3, are associated with heterochromatin and make the DNA more condensed and less accessible to the transcriptional machinery [93, 94]. Cytosine methylation is associated with increased H3K9me3 and decreased H3 acetylation and H3K4me3 at gene promoters, leading to chromatin condensation and subsequent transcriptional silencing [95, 96]. The level of histone modifications is orchestrated by histone modifying enzymes, which add or

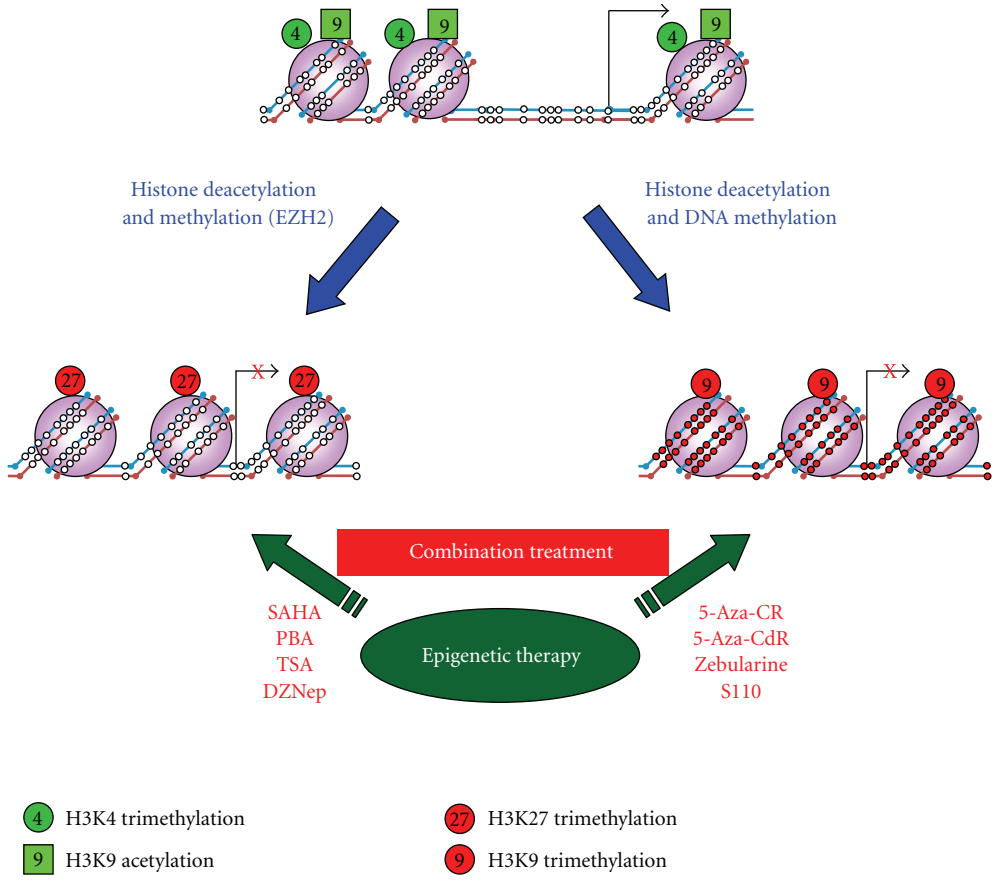

Figure 2: Epigenetic therapies can reverse aberrant epigenetic modifications in cancer. Genes that are expressed in normal cells, such as tumor suppressor genes, have an open chromatin structure, consisting of an unmethylated promoter, active histone marks, and a nucleosome-free region immediately upstream of the transcription start site. During tumorigenesis, genes can be silenced through one of the two silencing mechanisms: polycomb repressive complex (PRC) reprogramming and *de novo* DNA methylation. PRC-mediated silencing can be reversed upon treatment with EZH2 inhibitors, such as DZnep. The *de novo* methylation-mediated silencing can be reversed upon treatment with DNA methylation transferase inhibitors, such as 5-Aza-CdR, 5-Aza-CR, Zebularine, and S110. The therapeutic value of above reagents may be enhanced when combining with HDAC inhibitors, such as SAHA, PBA, and TSA. Open and closed circles represent unmethylated and methylated CpG sites, respectively.

remove specific histone marks to promote or hinder gene expression. A balance between these enzymes is necessary to maintain normal physiological conditions. Cancer cells lack this balance, as they typically overexpress histone deacetylases (HDAC), which results in a global reduction in histone acetylation [97].

More than 15 HDAC inhibitors are currently undergoing preclinical or clinical investigations for the treatment of both hematological malignancies and solid tumors, including UC [98]. Their common mechanism of action is the chelation of Zn^{2+} ion, which is critical to the enzymatic activity of HDAC [99]. To date, there are only 2 HDACIs that have been approved by the FDA for the treatment of cutaneous T-cell lymphoma, Vorinostat, also known as suberoylanilide hydroxamic acid (SAHA), and Romidepsin [94, 100]. HDACi have shown great clinical efficacy as single anticancer therapy only against certain hematological malignancies [101]. Although many have shown great potential for solid tumors in preclinical settings, in clinical settings they have generally yielded low responses [97, 102]. Among such HDACi, SAHA showed modest efficacy against UC in a phase I trial [103, 104]. To date, HDACi have demonstrated limited antitumor activity in UC and other solid tumors as a single agent; however, they have been well tolerated by patients [105]. *In vivo* studies have shown that a combinatorial treatment of HDACi and adenovirus-mediated gene therapy is more efficacious than either one alone, resulting in upregulation of the coxsackie and adenovirus receptor (CAR) gene, which is essential for the uptake of adenoviruses in target cells [106–108]. Such studies suggested the potential benefits of combining HDACi with other therapeutic agents to achieve a better therapeutic value in treating patients with UC.

9. Combination Therapy

The epigenome of UC is highly disrupted, featuring aberrant gene silencing either through the acquisition of DNA methylation or the repressive histone mark H3K27 trimethylation (Figure 2). The existence of these mechanisms suggests that

the combination of DNMTi and HDACi may result in higher therapeutic efficacy. Both additive and synergistic effects have been reported with the combination of these two classes of epigenetic agents in patients with advanced hematological malignancies and solid tumors [32, 102]. However, the clinical utilization of combined epigenetic therapies is still in its early stages and more work is needed to elucidate the mechanism behind the increased clinical efficacy of sequential administration of DNMTi and HDACi in order to achieve an even greater synergistic effect.

The discovery of the vital role that aberrant epigenetic changes play in tumorigenesis as well as the reversibility of such changes has spurred great interest in the application of epigenetic therapies in cancer treatment with the primary goal of restoring aberrantly silenced genes. In addition, epigenetic therapies can also enhance the expression of cancer germline antigens, which are genes only expressed in germ cells and in a variety of cancers, including UC [109, 110]. Activating such genes increases the likelihood that tumor cells will be recognized and killed by antigen reactive CD8(+) T cells [111]. Epigenetic therapy can enhance the expression of cancer germline antigens, which are being actively pursued as vaccine targets. Therefore, combining epigenetic therapy with cancer germline antigen vaccine therapy may help amplify the therapeutic value of immunotherapy [110].

Despite its great promise, the application of epigenetic therapies to the treatment of UC and other types of solid tumors is still in its infant stage. Some of the issues that need to be resolved before this therapeutic approach is implemented includes the poor stability of the two FDA-approved DNMTi and the relapse of methylation after DNMTi treatment.

10. Conclusion and Future Directions

UC is as much a disease of disrupted epigenome as it is a disease of genetic mutations. Here, we have summarized the epigenetic abnormalities associated with UC, with an emphasis on DNA methylation. The presence of an epigenetic field defect, where DNA methylation of a significant number of genes is altered not only in primary tumors but also in the surrounding normal-appearing tissues, provides a plausible explanation for the high rate of UC recurrence. Since certain epigenetic alterations precede disease pathology, they have the potential to serve as excellent biomarkers for diagnosis, prognosis, and monitoring. Although a large number of highly specific markers, both genetic and epigenetic, have already been identified, they suffer from low sensitivity. The ability to detect methylation changes in readily obtainable urine samples opens the door for the development of sensitive and specific noninvasive methods for early detection and monitoring of UC. In addition to serving as biomarkers, epigenetic alterations are also excellent therapeutic targets. Epigenetic therapies, such as DNMTi and HDACi, aim at restoring the diseased epigenome to its normal state by reactivating aberrantly silenced genes. While they have shown promising results in both preclinical and clinical settings, their efficacy is still limited to a few hematological malignancies. Epigenetic therapies also reactivate cancer germline antigens, which can be recognized by the immune system, and, therefore, they could potentially enhance the therapeutic value of cancer germline antigen vaccines. Future work, including obtaining a greater understanding of the mechanisms of DNMTi and HDACi, is necessary to determine the extent of their utility in treating solid tumors. With the aid of readily available genome-wide DNA methylation and expression analyses and our rapidly accumulating knowledge regarding epigenetic regulation, the translation of these findings from the bench to the bedside in the near future is an obtainable goal.

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Review Article

Bladder Cancer Immunotherapy: BCG and Beyond

Eric J. Askeland, Mark R. Newton, Michael A. O'Donnell, and Yi Luo

Department of Urology, University of Iowa, 375 Newton Road, 3204 MERF, Iowa City, IA 52242, USA

Correspondence should be addressed to Yi Luo, yi-luo@uiowa.edu

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Mycobacterium bovis bacillus Calmette-Guérin (BCG) has become the predominant conservative treatment for nonmuscle invasive bladder cancer. Its mechanism of action continues to be defined but has been shown to involve a T helper type 1 (Th1) immunomodulatory response. While BCG treatment is the current standard of care, a significant proportion of patients fails or do not tolerate treatment. Therefore, many efforts have been made to identify other intravesical and immunomodulating therapeutics to use alone or in conjunction with BCG. This paper reviews the progress of basic science and clinical experience with several immunotherapeutic agents including IFN-α, IL-2, IL-12, and IL-10.

1. Introduction

With more than 73,000 estimated cases diagnosed in 2012, bladder cancer is the fifth most common malignancy in the United States, responsible for more than 14,000 deaths per year [1]. Urothelial carcinoma accounts for 90% of bladder tumors, of which approximately 70% are confined to layers above the muscularis propria—the so-called nonmuscle invasive bladder cancer (NMIBC). These tumors (previously termed "superficial bladder tumors") include stages Ta, T1, and Tis, occurring in 70%, 20%, and 10% of NMIBC cases, respectively [2]. Standard primary treatment for NMIBC is transurethral resection (TUR); however, recurrence rates for TUR alone can be as high as 70% with up to 30% progressing to muscle invasive disease requiring cystectomy [3].

High rates of recurrence and progression have prompted investigation into a myriad of treatments attempting to decrease the burden of this disease. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the most well known and studied of these adjunctive treatments. Since its first description in 1976 by Morales et al. [4], intravesical BCG has become the standard therapy for NMIBC, superior to any other single chemotherapeutic agent for reducing recurrence and preventing progression. Typical complete response rates are 55–65% for papillary tumors and 70–75% for carcinoma *in situ* (CIS), which inversely indicates that 30–45% of patients will be BCG failures [5–7]. Of the complete responders, up to 50% will have a recurrence [8]. Furthermore, side effects range from cystitis and irritative voiding symptoms to much more uncommon life-threatening BCG sepsis. Up to 20% of patients are BCG intolerant due to these side effects [9].

Understanding of BCG, both its mechanisms (which remain incompletely characterized) and its obvious limitations, is critical to improving the efficacy of therapy. The initial step after BCG instillation is binding of BCG to fibronectin expressed on the urothelium, after which the *mycobacterium* is internalized by both normal and malignant cells, resulting in urothelial activation and subsequent inflammatory responses in the bladder [10]. BCG antigens can be presented at the cell surfaces of urothelial and antigen-presenting cells in the context of major histocompatibility complex (MHC) class II, stimulating $CD4^+$ T cells and inducing a primarily T helper type (Th) 1 immune response [11]. This complex and robust immune reaction evoked by BCG is evidenced by a massive transient secretion of cytokines in voided urine, including interleukin (IL)-1, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18, interferon-inducible protein (IP)-10, tumor necrosis factor (TNF)-α, granulocyte-monocyte colony stimulating factor (GMCSF), and interferon (IFN)-γ [12]. While the role each of these cytokines plays in urothelial carcinoma treatment is not

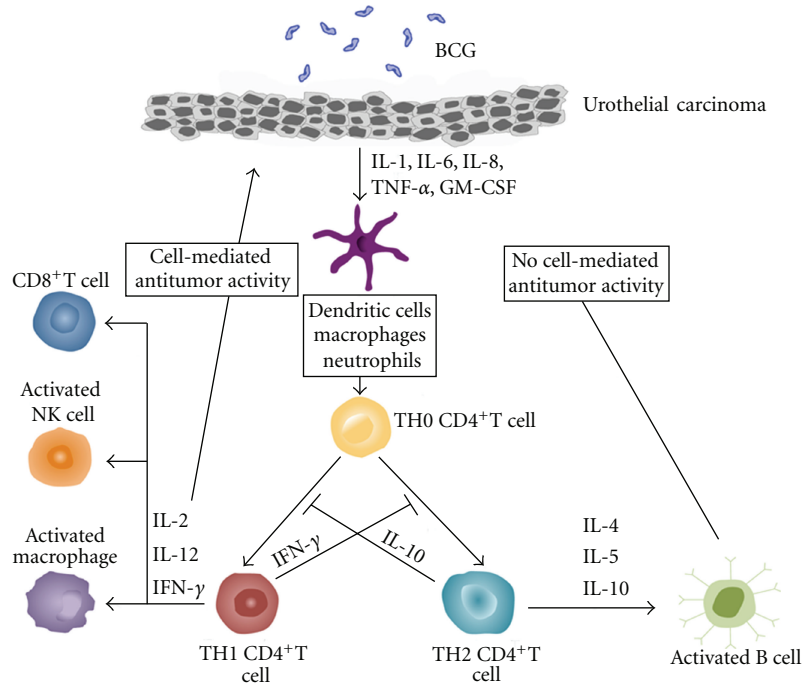

Figure 1: Suggested cascade of immune responses in bladder mucosa induced by intravesical BCG instillation. Attachment of BCG to urothelial cells including carcinoma cells triggers release of cytokines and chemokines from these cells, resulting in recruitment of various types of immune cells into the bladder wall. Activation of phagocytes and the new cytokine environment lead to the differentiation of naïve $CD4^+$ T cells into TH1 and/or TH2 cells that direct immune responses toward cellular or humoral immunity, respectively. The therapeutic effect of BCG depends on a proper induction of TH1 immune responses. IL-10 inhibits TH1 immune responses, whereas IFN-γ inhibits TH2 immune responses. Blocking IL-10 or inducing IFN-γ can lead to a TH1-dominated immunity that is essential for BCG-mediated bladder cancer destruction.

completely clear, Th1 cytokines (e.g., IFN-γ, IL-2, and IL-12) have been associated with BCG response, while Th2 cytokines (e.g., IL-10 and IL-6) correlate with BCG failure, as illustrated in Figure 1 [13–16]. Since the advent of BCG therapy, a significant amount of data has accumulated to support maintenance treatments, which typically consist of a series of shorter treatments at 3–6-month intervals, often based on the time table developed by the Southwest Oncology Group [17].

While success has improved with the addition of maintenance treatments, the combination of intravesical therapy, surveillance, and repeat surgical procedures place enormous costs on the US healthcare system, approaching $4 billion annually [3]. Prompted by the burden of patients either with BCG refractory disease or who are intolerant of treatment, the search goes on for therapeutic improvements. Given that the effect of BCG is immune mediated, decades of research have focused on adjunctive immunotherapies including IFN-α, IL-2, IL-10, and IL-12. This paper summarizes and integrates key points for the clinical urologic oncologist.

2. Interferon-α2b

Interferons (IFNs) are glycoproteins initially isolated in the 1950s and valued for their antiviral properties. Three types have been isolated, IFN-α (which is actually a family of interferons), IFN-β, and IFN-γ. IFN-α and IFN-β are grouped as "Type I" interferons, whereas IFN-γ is a "Type II" interferon. The Type I interferon receptor has 2 components, IFNAR-1 and IFNAR-2, which subsequently bind and phosphorylate Jak molecules initiating a cascade resulting in gene transcription [18]. The IFN-α family is well known to stimulate natural killer (NK) cells, induce MHC class I response, and increase antibody recognition [19]. They have antineoplastic properties by direct antiproliferative effects and complex immunomodulatory effects [18], both of which could be advantageous for bladder cancer treatment. Clinically available preparations include IFN-α2a (Roferon-A, Roche Laboratories, Nutley, NJ) and IFN-α2b (Intron-A, Schering Plough, Kenilworth, NJ), though to date most research involves IFN-α2b. There has been interest in IFN-α2b both alone and in combination with BCG, where a synergistic response has been described. Conceptually, combining BCG and IFN makes sense. BCG efficacy depends on the induction of a robust Th1 cytokine profile, and IFN-α2b has been shown to potentiate the Th1 immune response [12]. However, despite theoretical promise, data after translation to clinical practice has been mixed.

For many years, IFN-α was thought to exert antitumor activity primarily through direct antiproliferative properties [20]. At least part of this effect has been shown to be

mediated by directly inducing tumor cell death. IFN-α has been documented to independently induce Tumor-Necrosis-Factor-Related Apoptosis-Inducing Ligand (TRAIL) expression in UM-UC-12 bladder cancer cells [21], which subsequently triggers apoptosis in cells expressing the appropriate cell death receptor. Cell death occurs ultimately by Fas-associated protein with death-domain- (FADD-) dependent activation of the death inducing signaling complex (DISC) followed by activation of caspase-8. Furthermore, Tecchio and colleagues have demonstrated that IFN-α can stimulate TRAIL mRNA as well as the release of a bioactive soluble TRAIL protein from neutrophils and monocytes, which induces apoptotic activity on TRAIL-sensitive leukemic cell lines [22]. It also appears that IFN-α apoptotic effects may not be limited to TRAIL; rather it may trigger caspase-8 via both cell death receptor-dependent and independent pathways [23]. Much like IFN-α, BCG has also been shown to induce TRAIL [24], which has correlated with patient response to BCG therapy and has been a source of overlapping research interest. Other direct IFN-α effects include enhancing cytotoxicity of $CD4^+$ T cells, increasing antigen detection by upregulating MHC class I expression [20, 25, 26]. Direct suppression of proliferation by induction of tumor suppressor genes or inhibition of tumor oncogenes has also been described [20]. Also contributing to antiproliferative properties, IFN-α has been documented to decrease angiogenesis and basic fibroblast growth factor. Additionally, it downregulates matrix metalloprotease-9 (MMP-9) mRNA as well as the MMP-9 translational protein in murine bladder tumors [27]. Interestingly, it has also been demonstrated that an optimal biologic dose with higher frequency, rather than maximal tolerated dose, produced the most significant decreases in angiogenesis. Significantly decreased angiogenesis has also been documented in human urothelium during and after IFN-α2b treatment following transurethral resection of superficial bladder tumors [28].

In vivo monotherapy with IFN-α2b for bladder cancer in humans has been explored by multiple groups. In 1990, Glashan published data from a randomized controlled trial evaluating high dose (100 million unit) and low dose (10 million unit) IFN-α2b regimens in patients with CIS [29]. Patients were treated weekly for 12 weeks and monthly thereafter for 1 year. The high and low dose groups had complete response rates of 43% and 5%, respectively. Of the high dose patients achieving a complete response, 90% remained disease-free at a notably short 6 months of follow-up. The primary side effects of treatment were flu-like symptoms (8% low dose, 17% high dose) but without the irritative symptoms seen so often in BCG therapy. When IFN-α2b was investigated alone to treat BCG failures, eight of twelve patients had recurrence at initial three-month evaluation and only one of twelve was disease-free at 24 months [30]. Another trial conducted by Portillo and colleagues randomized 90 pT1 bladder cancer patients to either intravesical treatment or placebo groups as primary prophylaxis after complete resection [31]. They utilized a similar dosing schedule but used 60 million units IFN-α2b. At 12 months of followup, recurrence rates were significantly lower for IFN-α2b group than placebo, 28.2% versus 35.8%, respectively. However, after 43 months, rates were similar—53.8% and 51.2%, respectively, indicating that treatment benefit of IFN-α2b alone may not be durable.

Given the described antiproliferative and immunomodulatory effects of IFN-α, combination therapy with BCG has held tantalizing promise. Gan et al. found significantly greater antitumor activity with combination therapy than BCG alone: 14/15 mice receiving BCG/IFN-α versus 8/15 mice receiving only BCG became tumor-free after 5 weekly intralesional treatments [32]. In an *in vitro* study comparing BCG plus IFN-α to BCG alone, our group demonstrated a 66-fold increase in IFN-γ production in peripheral blood mononuclear cell (PBMC) cultures [12]. Since IFN-γ is a major Th1-restricted cytokine found in patients responding to BCG therapy, it has been used routinely as a surrogate marker for Th1 immune response in studies examining effect of IFN-α [12]. It appears that IFN-α by itself generates a negligible Th1 response, as no significant levels of IFN-γ were detected after IFN-α was incubated alone with the PBMCs. We have also demonstrated that the augmented IFN-γ production persisted even with reduced doses of BCG. These findings give credence to the idea that adding Th1-stimulating cytokines may allow for a decrease in BCG doses, thereby decreasing side effects thought to be directly related to BCG. Further augmenting Th1 differentiation, IFN-α was found to increase levels of several Th1 cytokines, including IL-12 and TNF-α as well as decreasing known Th1 inhibitory cytokines IL-10 and IL-6 by 80–90% and 20–30%, respectively [33].

Clinical investigations with the combination of IFN-α2b and BCG began initially in BCG refractory patients but were subsequently expanded to BCG naïve patients. Stricker et al. found the combination to be safe, with a similar side effect profile to BCG alone [34]. In 2001, O'Donnell and colleagues reported on combination therapy administered to 40 patients who had failed at least 1 course of BCG alone [35]. At 24 months, 53% of patients were disease-free. Patients with two or more prior BCG failures faired similarly to patients with only one. Lam et al. in 2003 reported on the treatment of 32 patients, of which 20 (63%) were BCG failures. At 22 months' median followup, 12 of the 20 BCG failure patients (60%) remained disease-free [36]. In a smaller trial, Punnen et al. documented a 50% disease-free rate after combination therapy at 12 months' followup in 12 patients with BCG refractory disease [37]. A subsequent large community-based phase II clinical trial examined 1106 patients from 125 sites with NMIBC, which were split into BCG naïve and BCG refractory groups [38]. At median 24 months' followup, tumor-free rates were 59% and 45%, respectively. In this larger trial, patients who had two or more courses of prior BCG therapy had a worse outcome when compared to patients who had 1 or less, likely indicating more resistant disease. A recent study limited to BCG naïve patients demonstrated similar disease-free rate of 62% but with much longer median followup of 55.8 months [39]. Furthermore, after evaluating failure patterns and response rates to BCG plus IFN-α, Gallagher et al. found that patients who recurred more than 12 months after initial BCG treatments had similar tumor-free rates at 24 months when compared to

BCG naïve patients [40]. However, patients who recurred within a year of receiving their initial BCG treatments did significantly worse, with disease-free rates of 34–43% at 24 months, indicating that additional immunotherapy may not be appropriate. Overall, while promising, these data are unable to define any treatment benefit of combination therapy over BCG alone.

To date, the only randomized trial comparing BCG alone to BCG plus IFN-α was a multicenter study of 670 BCG naïve patients with CIS, Ta, or T1 urothelial carcinoma [41]. This was a four-arm trial evaluating efficacy of megadose vitamins as well as BCG and IFN. Patients were randomized to 1 of 4 groups: BCG plus recommended daily vitamins, BCG plus megadose daily vitamins, BCG plus IFN-α2b plus recommended daily vitamins, and BCG plus IFN-α2b plus megadose daily vitamins. At 24-month followup, median recurrence-free survival was similar across all groups, though the two IFN-α2b groups experienced higher incidence of constitutional symptoms and fever ($P < 0.05$).

Lastly, there are multiple areas where additional research is warranted. A recent evolution in combination therapy has been the development of an IFN-α2b expressing strain of recombinant BCG (rBCG-IFN-α) from the Pasteur strain of BCG. An initial *in vitro* study documented enhanced IFN-γ expression in PBMCs after incubation with rBCG-IFN-α as compared to standard BCG [42]. A subsequent study reported that rBCG-IFN-α increased cytotoxicity up to 2-fold over standard BCG in PBMC cultures. Both $CD56^+CD8^-$ NK cells and $CD8^+$T cells were identified as primary contributors to the increased cytotoxicity [43]. Combining IFN-α2b with other antiproliferative agents has shown *in vitro* promise. Louie et al. reported that a combination of IFN-α2b and maitake mushroom D-fraction (PDF) could reduce T24 bladder cancer cell proliferation by 75%, accompanied by G_1 cell cycle arrest [44]. Another combination recently published this year documented that adding grape seed proanthocyanin significantly enhanced antiproliferative effects of IFN-α2b, with >95% growth reduction in T24 bladder cancer cells. Cell cycle analysis also revealed G_1 cell cycle arrest, with Western blots confirming expression of G_1 cell cycle regulators [45]. Lastly, several groups have investigated gene therapy with a recombinant adenovirus delivery system (rAd-IFN/Syn3), which could potentially result in sustained therapeutic IFN-α2b levels for long periods of time. Nagabhushan et al. were able to demonstrate delivery and expression of IFN in the bladder as well as significant tumor regression in mice. Phase I trials with rAd-IFN/Syn3 were ongoing at the time of their publication in 2007 [46].

3. Interleukin-2

The discovery and characterization of interleukin-2 (IL-2) was one of the most important breakthroughs in the field of immunology. Prior to its discovery, lymphocytes were thought to be terminally differentiated and incapable of proliferation [47, 48]. In 1975, it was discovered that the supernatant of murine splenic cell cultures could stimulate thymocytes, suggesting a native effector protein was responsible for this mitogenic activity [48, 49]. When initially examined independently by different investigators, this "effector protein" was given multiple working names including thymocyte-stimulating factor (TSF), thymocyte mitogenic factor (TMF), T cell growth factor (TCGF), costimulator, killer cell helper factor (KHF), and secondary cytotoxic T-cell-inducing factor (SCIF) [50]. In 1979, it was recognized that these factors likely represented the same entity, and the nomenclature was standardized with the term "interleukin" (between leukocytes). Thus, the "effector protein" was named IL-2, differentiating it from the only other interleukin known at that time, IL-1 [50]. Regardless of the nomenclature, this protein was recognized to promote proliferation of primary T cells *in vitro*, which revolutionized the experimental armamentarium in the field of immunology [47, 49, 51].

Since the discovery of IL-2-mediated control of T-cell growth in culture, there has been much progress in elucidating its mechanisms. It was discovered relatively early that IL-2 enhances the production of cytotoxic lymphocytes which are capable of lysing tumor cells while leaving normal cells unharmed [51–53]. These IL-2 activated lymphocytes became known as "lymphokine-activated killer" (LAK) cells and were thought to play a large role in antitumor immune function [51–53]. Additionally, it was noted that IL-2 functions to augment the cytotoxic activity of NK cells and monocytes [54, 55]. It has even been discovered that IL-2 is important for the activation of B cells [56]. As the $CD4^+$ Th1 and Th2 cell cytokine profiles were defined, it became clear that IL-2 is predominantly a Th1-secreted cytokine [57].

The cytotoxic antitumor capabilities induced in lymphocytes by IL-2 make it a potential cancer immunotherapeutic agent. To date, multiple studies have demonstrated regression of metastatic disease following systemic IL-2 treatment in some cancers [58]. Rosenberg et al. reported on 157 patients with a heterogenous mix of metastatic cancers refractory to other treatments including renal cell, colon cancer, breast cancer, and lymphoma. Patients were treated with either IL-2 and LAK cells or IL-2 alone. Between the two groups, 9 complete and 20 partial responses were obtained. Significant morbidity has been reported with systemic IL-2 much of which is secondary to increased capillary permeability [58, 59] and includes weight gain, hypotension, oliguria, elevated creatinine, and bilirubin. These tend to resolve with cessation of IL-2 therapy [58]; however, Rosenberg reported 4 treatment-related deaths among their 157 patients. Despite the reports of morbidity, IL-2 seemed to offer hope to patients with few treatment options.

With regard to bladder cancer, interest was stimulated after multiple investigators identified elevated IL-2 levels (as well as other cytokines) in urine of patients following BCG, suggesting an immunomodulatory effect of BCG [60–67]. Additionally, an elevation in IL-2 receptor expression has been documented on T cells in voided urine after BCG therapy [64, 66]. Increased levels of urinary IL-2 have also been found to correlate with BCG response, which supports the concept that a Th1 cytokine profile confers a favorable response to BCG [15]. Furthermore, elevated IL-2 has been

reported in the serum of patients following BCG instillation, which suggests both a local and systemic immune response to therapy [68, 69]. These findings led to the conclusion that IL-2 may have a therapeutic use in bladder cancer.

One of the first clinical trials reported evidence of bladder tumor regression following intralesional injections of IL-2, with no adverse events recorded [70]. Multiple murine studies have demonstrated that systemic administration of IL-2, with or without BCG, can significantly decrease tumor size, suppress tumor growth, and improve mean survival [71–73]. A small clinical study investigating systemic IL-2 administration effects on low-stage bladder cancer found a complete and partial response rate in 5 of 12 patients, though 2 patients discontinued therapy due to toxicity [74]. The poor side effect profile of systemic IL-2 administration subsequently prompted a shift to utilize IL-2 as an intravesical therapy. Reports of intravesical use revealed a much improved side effect profile as well as some efficacy alone or when combined with BCG [75–78]. Den Otter et al. administered intravesical IL-2 alone after incomplete transurethral resection of grade 1-2, T1 papillary urothelial carcinoma, and documented "marker lesion" regression in 8 of 10 patients [79]. Additional experiments have focused on developing recombinant-IL-2-secreting strains of BCG [80–85]. Animal models using this approach have shown that compared to native BCG, IL-2-secreting BCG strains have increased IFN-γ production, induced a more favorable IFN-γ to IL-4 ratio, improved antigen-specific proliferation, enhanced antitumor cytotoxicity, and mounted a Th1 cytokine profile even in immunosuppressed or IL-4 transgenic mice (two conditions which favor a Th2 response) [80–83, 85]. More recent animal and *in vitro* studies have investigated IL-2 transfecting dendritic cells (DCs), immobilized streptavidin-tagged bioactive IL-2 on the biotinylated surface of murine bladder mucosa, and development of a murine IL-2 surface modified bladder cancer vaccine [86–89]. Since IL-2 plays a crucial role in the Th1 response, it will continue to be a source of interest for immunotherapy of bladder cancer.

4. Interleukin-12

Interleukin-12 (IL-12) has been the focus of significant cancer research among cytokines as well. In 1987, it was discovered through *in vitro* experiments that there existed a factor which synergized with IL-2 in promoting a cytotoxic T lymphocyte (CTL) response [89]. This factor was given the name cytotoxic lymphocyte maturation factor (CLMF) [89]. Shortly thereafter a factor was discovered that induced IFN-γ production, enhanced T cell responses to mitogens, and augmented NK cell cytotoxicity [90]. This factor was provisionally called natural killer cell stimulatory factor (NKSF) [90]. It did not take long to discover that these factors represented the same entity, thus the nomenclature converged and this protein was termed IL-12 [91–95].

Although initially discovered in a B cell lymphoma, it was subsequently found that IL-12 is primarily involved with the regulation of T cells, causing proliferation of both activated $CD4^+$ and $CD8^+$ T cell subsets while causing minimal proliferation of resting PBMCs [90, 92]. This concept is supported by studies demonstrating that the IL-12 receptor is upregulated in activated T and NK cells, but not in activated B cells [95]. IL-12 potentiates a Th1-specific immune response, and it was later discovered that DCs produce IL-12 and thus direct the development of Th1 cells from naïve $CD4^+$ T cells [96, 97]. Additionally, IL-12 can, by itself, stimulate the activation of nonspecific LAK cells and facilitate the generation of an allogeneic CTL response [98]. IL-12 has even been found to play a role in the activation of neutrophils [99, 100]. Multiple studies have demonstrated that IL-12 strongly inhibits neovascularization, thought to be mediated through its induction of IFN-γ [101–104]. Furthermore, the mechanism by which IL-12 enhances the cytolytic effect of NK cells is primarily via the perforin pathway [105, 106].

Multiple animal studies have shown tumor responsiveness to immunomodulation with IL-12. Using systemic or peritumoral injections, IL-12 showed antitumor properties in murine sarcoma, melanoma, renal cell carcinoma, lung cancer, colon cancer, breast cancer, and bladder cancer models [102, 107–111]. Increases in serum IFN-γ were observed in mice treated with IL-12 [108]. Antitumor efficacy was lost in $CD8^+$-depleted mice, but not $CD4^+$-depleted mice or NK-deficient mice, suggesting that the primary mediators of the antitumor IL-12 effect are $CD8^+$ T cells [107, 108]. Some of these studies saw effectiveness even with metastatic disease, including bladder cancer [107, 108, 111]. Multiple murine studies have also revealed added effectiveness with IL-12 administered in combination with chemotherapeutic agents [109, 112–114]. Additionally, IL-12 therapy has shown synergistic activity when combined with radiation therapy in mice [110, 115]. Various delivery systems for IL-12 therapy have been tested in mice using viral and retroviral vectors to elicit an IL-12 response [116–120]. These constructs have shown some effectiveness as anticancer therapeutics [116–119]. IL-12 as an intravesical therapy for bladder cancer has shown great success in mouse models. BCG was found to be a potent stimulus for IL-12 expression, and neutralization of IL-12 significantly dampened the induction of IFN-γ by BCG [121]. BCG therapy for murine bladder cancer was essentially found to be ineffective in IL-12 knock-out mice, suggesting a crucial role for IL-12 in the BCG response [122]. When IL-12 is used as a therapy with BCG, it causes a synergistic induction of IFN-γ [121]. Intravesical IL-12 treatment alone was found to be effective for the treatment of orthotopically placed bladder tumors in mice, and urinary IFN-γ was subsequently found to be significantly elevated [111, 123]. These observations further support the importance of IFN-γ induction for effective immunotherapy of bladder cancer. More recently, multiple attempts have been made to improve the delivery of intravesical IL-12 to the bladder mucosa to improve efficacy. One method utilized cationic liposome-mediated IL-12 gene therapy, which showed improved survival and tumor-specific immunologic memory in mice [124]. Another method utilized chitosan, a mucoadhesive biopolymer, to increase IL-12 delivery to urothelial surfaces [125]. This method showed improved efficacy over IL-12 alone in a mouse model [125].

The great success of IL-12 in treating various murine cancers subsequently led to experiments testing its use on human cancers, though with mixed success. Initial trials focused on systemic IL-12 treatment for metastatic cancer, though progress was initially halted when several patients suffered severe toxic effects from the treatment and two patients died from the therapy [126]. A phase I trial of systemically administered IL-12 in 40 patients with advanced malignancy found a dose-dependent increase in circulating IFN-γ with administration [127]. Experiments on the peripheral blood of these patients showed augmented NK cell cytolytic activity and enhanced T cell proliferation [128]. Unfortunately, of these 40 patients there was only one partial response and one transient complete response [127]. Further studies looking at chronic administration of twice weekly IL-12 in patients with metastatic cancer found that it is well tolerated and induces costimulatory cytokines (including IFN-γ) [129]. However, in a cohort of 28 patients, there was only one patient with a partial response and two with prolonged disease stabilization, with one of these patients eventually exhibiting tumor regression [129]. Similar low response rates have been seen with systemic IL-12 in other studies of advanced malignancies [130–134]. Various combinations of immunotherapy have been tested with systemic IL-12 in humans. A phase I study examined systemic IL-12 with low dose IL-2 and showed it was well tolerated, and the addition of IL-2 significantly augmented IFN-γ production as well as the NK response [135]. Of 28 patients, there was one partial response and two pathologic responses [135]. Another phase I study using systemic IL-12 with IFN-α2b showed acceptable toxicity, but with no response in 41 patients [136]. As discussed previously, intravesical IL-12 showed great promise for the topical treatment of bladder cancer in a mouse model; however, this success did not translate clinically in humans. A phase I study of intravesical IL-12 therapy in patients with superficial bladder cancer showed minimal toxicity, but disappointing efficacy [137]. A total of 15 patients were enrolled in this study, of which 12 had no visible pretreatment lesions [137]. Of these 12 patients, 7 remained disease-free and 5 had recurrence within 4 weeks. The remaining 3 patients with pretreatment lesions had persistent disease at followup [137]. Perhaps the most disparaging results were that there was negligible IFN-γ-induced in the urine and serum of these patients post-treatment, suggesting minimal immunologic effect from intravesical IL-12 therapy [137]. Despite the disappointing results from human studies, IL-12 remains an important target for the treatment of bladder cancer.

5. Interleukin-10

Unlike other cytokines previously discussed, interleukin-10 (IL-10) is distinct in that its primary effect is to promote a Th2 response and thus dampen the immunotherapeutic effects of BCG for the treatment of bladder cancer [54, 138, 139]. As a result, it may have therapeutic value not by its native function, but by abrogation of its native function. IL-10 was first characterized in 1989. It was initially termed cytokine synthesis inhibitory factor (CSIF), a rather fitting name, because it was found to inhibit the production of several cytokines produced by Th1 clones [140]. The most important of these cytokines was IFN-γ, which was recognized as an important player in the Th1 response. As discussed previously, it is a key contributor in the immunotherapeutic effectiveness of BCG [140, 141]. Further studies showed that IL-10 prevented a delayed-type hypersensitivity (DTH) response to BCG and the neutralization or abrogation of IL-10 prolonged a response, thus further supporting its role in the Th1/2 response [138, 142]. Several human tumors, including melanoma, non-small-cell lung carcinoma, renal cell carcinoma, and bladder cancer, have been found to express elevated levels of IL-10 [143–147]. It is speculated that production of IL-10 by tumor cells may represent an "escape mechanism" whereby tumor cells avoid Th1-immune-mediated tumoricidal effects [143].

There has been significant progress in determining the regulation and mechanism of IL-10 function since its discovery, particularly with regard to its role in tumor immunology. It is secreted by multiple cell types including Th2 cells, B cells, and monocytes/macrophages [140, 148–150]. Like many other cytokines, IL-10 is known to autoregulate itself by downregulating its own mRNA synthesis [150]. It has been shown to block the accumulation of macrophages and DCs at tumor sites, which are important players in the cellular immune response [151, 152]. Additionally, it compromises DCs ability to stimulate T-cells causing induction of antigen-specific anergy of T cells [153]. Furthermore, it down-regulates the expression of MHC class II and costimulatory molecules, thus preventing a cellular immune response to tumor cells [154–156]. During activation of $CD4^+$ T cells, the presence of IL-10 can cause them to differentiate into T regulatory cells 1 (Tr1), leading to peripheral tolerance [157]. IL-10 further reduces cellular tumoricidal activity by preventing release of reactive nitrogen/oxygen intermediates by macrophages and NK cells, a key step in their efficacy during cellular immune defense [139, 158].

Successful treatment of bladder cancer with BCG, as discussed previously, requires a Th1 cytokine profile. IL-10 antagonizes the production of a Th1 milieu, thus its neutralization has been targeted as a potential means to augment the BCG response. Several murine studies have demonstrated that after IL-10 knockout mice are inoculated with bladder cancer, they have an improved BCG and local immune response, increased bladder mononuclear infiltrate, enhanced DTH responses, greater antitumor activity, and prolonged survival [54, 138, 143]. Although murine IL-10 knockout studies are conceptually important, studies focused on IL-10 neutralization hold more promise as clinically useful therapeutics. Murine bladder cancer studies utilizing anti-IL-10 neutralizing antibody have shown similar results, with BCG treatment inducing an enhanced DTH response and increased bladder mononuclear infiltrate [138, 142]. More recent efforts have been placed at targeting the IL-10 receptor. The IL-10 receptor is composed of two known subunits (IL-10R1 and IL-10R2), and the IL-10R1 subunit plays the predominant role in signal transduction [159]. In *in vitro* studies, we have recently shown that splenocytes incubated with BCG and anti-IL-10-receptor 1 monoclonal

antibody (anti-IL-10R1 mAb) produced significantly higher IFN-γ than those incubated with BCG plus anti-IL-10-neutralizing antibody, suggesting that interference with IL-10 signal transduction may be more effective than neutralizing IL-10 protein (17). In *in vivo* studies, mice treated with BCG and anti-IL-10R1 mAb showed increased urinary IFN-γ production compared to BCG controls [160]. In a similar murine experiment, there was improved overall and tumor-free state in mice treated with BCG plus anti-IL-10R1 mAb compared to BCG treatment controls, though this difference did not reach statistical significance [160]. Most recently, in an experiment designed to follow murine survival after inoculation with a luciferase-expressing MB49 bladder cancer cells, we discovered that control mice and BCG-only treated mice developed histologically confirmed lung metastasis, whereas mice treated with BCG and anti-IL-10R1 mAb developed no metastasis [unpublished data]. This difference was statistically significant and raises questions as to anti-IL-10R1 mAb's role as more than just an augmentation to BCG for local bladder cancer control. Confirmatory experiments and mechanistic studies are necessary, but anti-IL-10R1 mAb shows great potential in not only local bladder cancer control, but also possibly systemic immunomodulation for the prevention of metastatic bladder cancer.

6. Conclusions

Bladder cancer is a disease that places significant social and financial burdens both on the patient and on society, costing nearly $4 billion annually in the U.S. BCG, which stimulates a robust immune response in most patients and has become the standard of care after surgical resection of nonmuscle invasive disease. However, despite treatment, a significant portion of patients still recur or are intolerant of BCG side effects. Multiple immunotherapies including IFN-α, IL-2, IL-12, and IL-10 have been investigated, either as adjuncts with BCG or as a solo replacement therapy. To date, there are a multitude of encouraging *in vitro* and murine studies; however, no clinical data has yet been reported, which is compelling enough to change the standard of care, yet many practitioners continue to use adjunctive immunotherapy based on basic science data and theoretical benefit. At our institution, for instance, BCG or BCG/IFN-α refractory disease is often treated with "quadruple therapy"—a combination of BCG, IFN-α, IL-2, and GM-CSF. The widespread use of immunotherapy for bladder cancer highlights the need for additional basic science and clinical research to further our understanding of tumor biology, human immunology, and the treatment of urothelial carcinoma.

Authors' Contribution

E. J. Askeland and M. R. Newton contributed equally to the production of this paper.

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Review Article

Methylation Markers for Urine-Based Detection of Bladder Cancer: The Next Generation of Urinary Markers for Diagnosis and Surveillance of Bladder Cancer

Thomas Reinert

Department of Molecular Medicine, Aarhus University Hospital, 8200 Aarhus N, Denmark

Correspondence should be addressed to Thomas Reinert, thomas.reinert@ki.au.dk

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Cancer of the urinary bladder is the fifth most common neoplasm in the industrialized countries. Diagnosis and surveillance are dependent on invasive evaluation with cystoscopy and to some degree cytology as an adjunct analysis. Nomuscle invasive bladder cancer is characterized by frequent recurrences after resection, and up to 30% will develop an aggressive phenotype. The journey towards a noninvasive test for diagnosing bladder cancer, in order to replace or extend time between cystoscopy, has been ongoing for more than a decade. However, only a handful of tests that aid in clinical decision making are commercially available. Recent reports of DNA methylation in urine specimens highlight a possible clinical use of this marker type, as high sensitivities and specificities have been shown. This paper will focus on the currently available markers NMP22, ImmunoCyt, and UroVysion as well as novel DNA methylation markers for diagnosis and surveillance of bladder cancer.

1. Introduction

Cancer of the urinary bladder is the fifth most common neoplasm in the industrialized countries and in the Unites States, with an estimated 70,530 new cases of bladder cancer diagnosed and with 14,680 deaths in 2010 [1]. Risk factors associated with the development of bladder cancer are mainly smoking and to a lesser extent workplace exposure to carcinogens [2, 3]. No genomic risk markers have been discovered apart from a few SNPs with a very low increase in relative risk [4].

In approximately 70% of all cases the patients will present with nonmuscle invasive bladder cancer (NMIBC) of stages Ta, T1, or Tis, whereas the remaining 30% of the tumors will be muscle invasive stage T2–4 bladder cancers (MIBC). Tumor recurrences are frequent (70%) in patients with NMIBC, whereas progression to MIBC is less frequently observed (10%–30%) [5]. The standard treatment of NMIBC is transurethral resection (TUR) complemented by use of intravesical immunotherapy or chemotherapy in order to preclude recurrence and progression [6]. Risk factors associated with recurrence are tumor size, multiplicity, stage, and grade, whereas the risk factors for progression are tumor size, multiplicity, stage, high grade, and the presence of carcinoma in situ (CIS) [7]. The sensitivity of cystoscopy for NMIBC is close to 80% for white light cystoscopy and 96% when using hexaminolevulinate (HAL). The sensitivity of white light cystoscopy decreases to 48% and 68% for detection of dysplasia and CIS, respectively, whereas the sensitivity of cystoscopy using HAL for these lesions remains in the range of 93% to 95% [8, 9]. The high recurrence rate requires frequent and prolonged surveillance by cystoscopy and makes bladder cancer the most expensive cancer to treat overall [10, 11]. According to the EAU Guidelines on NMIBC, the current treatment regime for low risk NMIBC patients is cystoscopy at 3 months after TUR, and if negative, then the next cystoscopy is advised done 9 months later and then yearly for 5 years [6]. For high risk NMIBC and CIS patients the standard surveillance is cystoscopy and urinary cytology at 3 months, and if negative, this is repeated every three months for 2 years, then half-yearly for up to 5 years, and then yearly [6]. In patients with an intermediate risk of progression, surveillance is in between that advised for the followup for low- and for high-risk patients [6]. Cytology is a noninvasive

Table 1: Sensitivity and specificity of cytology, NMP22, ImmunoCyt, and UroVysion when no distinguishment is made between patients with suspicion of bladder cancer and patients previously diagnosed with bladder cancer.

Test	Samples/studies	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Interference by other bladder conditions	Comments	Reference
Cytology[a]	14260/36	44 (38–51)	96 (94–98)	Yes	Subjective judgement	[12]
NMP22[b]	10119/28	68 (62–74)	79 (74–84)	Yes	Cutoff was ≥10 U/mL for positive test result	[12]
ImmunoCyt	2896/8	84 (77–91)	75 (68–83)	Yes	At least one green or one red fluorescent cell	[12]
UroVysion	2535/12	76 (65–84)	85 (78–92)	No	Cutoffs may vary between studies	[12]

[a]Voided urine only.
[b]Including five studies using NMP22 BladderChek.

operator-dependent test often used in combination with cystoscopy, owing to a very high specificity of 96% (94–98; 95% CI), but with a low average sensitivity of 44% (38–51, 95% CI). Cytology has higher sensitivity for high-stage and high-grade tumors than for low-stage and low-grade tumors [12].

The perfect urinary marker of bladder cancer should reliably detect all tumors for the well-being of the patients and at the same time have a very high specificity to minimize false positive results. This is especially true when considering screening in general, but also when considering screenings of high risk groups and patients under suspicion of bladder cancer. Six markers are approved by the U.S. Food and Drug Administration (FDA) for diagnosis of bladder cancer in patients suspected of having bladder cancer, or for surveillance of bladder cancer. While they are all more sensitive than cytology, they are unable to replace cystoscopy. This paper will list the performance of cytology, the FDA-approved biomarkers NMP22, ImmunoCyt, and UroVysion, and investigational methylation urinary markers for the diagnosis and surveillance of bladder cancer. The performance of methylation markers will be compared with cytology and the FDA-approved markers. Furthermore, this paper will discuss whether the urinary methylation markers may replace cystoscopy, replace cytology as an adjunct to cystoscopy, or postpone the time between cystoscopies in the surveillance of NMIBC. Studies included in the review have been chosen based on the number of participants included, and the ability to determine whether primary lesions or recurrent lesions have been studied in the papers.

2. Cytology

Urinary cytology is the gold standard for noninvasive urinary diagnosis of bladder cancer, and the cytological examination is performed by a pathologist or cytologist who identifies cancer cells in the urine. The cells in the urine are classified into one of four categories: normal, atypical/indeterminate, suspicious, or malignant [13]. Urinary infections or other inflammatory conditions of the bladder may produce false positive results, and in addition the inter- and intraobserver variability of cytology are both high [14, 15]. Cytology has an overall test sensitivity of 44% (38–51, 95% CI) (Table 1), but low-grade tumors (grades I and II) have a lower test sensitivity than high-grade tumors. The test sensitivity of low-grade tumors is 27%, with a range from 0% to 93%, whereas high-grade tumors have a test sensitivity of 69%, with a range from 0% to 100%. Pooled estimates from 36 studies including 14,260 patients have established that the specificity of cytology is very high 96% (94–98; 95% CI) with a low false-positive interpretation [12]. In patients with a history of NMIBC the sensitivity is 38% (12–47; range) and the specificity is 94% (83–97; range) (Table 2) [12].

3. Commercially Available Bladder Cancer Markers

3.1. Nuclear Matrix Protein 22. The nuclear matrix protein 22 (NMP22) is a nuclear matrix protein that is found in all cells. During mitosis, the NMP 22 protein is found in the mitotic spindle, where it has an important role in the distribution of chromatin to daughter cells [16, 17]. The level of NMP22 in bladder cancer cell lines has been shown to be 25-fold more concentrated than in the healthy urothelium from a normal bladder [18]. In patients with bladder tumors, the NMP22 level was reported as 5 times greater than in individuals with no bladder malignancies [19]. False positive tests have been observed in individuals with benign inflammatory conditions, and several other nonmalignant conditions [20]. NMP22 was initially developed as a quantitative assay (NMP22 test), but was later transformed into the NMP22 BladderChek kit. The sensitivity and specificity of the NMP22 tests, counting 10,119 patients in 28 studies, are 68% (62–74; 95% CI) and 94% (83–97; 95% CI), respectively (Table 1). The NMP22 test has a higher sensitivity for high-grade tumors than for low-grade tumors. The test sensitivity for low-grade tumors is 50%, with a range from 0% to 86%, whereas the test sensitivity is 83%, with a range from 0% to 100%, for high-grade tumors [12]. In patients with a history of NMIBC, the test sensitivity is 69% (50–85; range) and the specificity is 81% (46–93; range) (Table 2) [12].

3.2. ImmunoCyt. ImmunoCyt combines cytology with an immunocytofluorescence technique based on a cocktail of monoclonal antibodies labeled with fluorescent markers. The monoclonal antibodies recognize a high molecular weight form of the carcinoembryonic antigen and two bladder tumor cell-associated mucins. The analysis is performed in

Table 2: Performance of cytology and biomarkers in studies that include patients with suspicion of bladder cancer or patients previously diagnosed with bladder cancer.

Test/marker	Suspicion[a]/previous[b] history of BC	Samples/ studies	Sensitivity, % Median (range)	Specificity, % Median (range)	Reference
Cytology	Suspicion	3331/7	44% (16–100)	96 (94–98)	[12]
	Previous history of BC	4495/6	38% (12–47)	94 (83–97)	
NMP22	Suspicion	1893/4	71 (56–100)	86 (80–87)	[12]
	Previous history of BC	4284/7	69 (50–85)	81 (46–93)	
ImmunoCyt	Suspicion	280/1	85	88	[12]
	Previous history of BC	326/1	81	75	
UroVysion	Suspicion	497/1	69	78	[12]
	Previous history of BC	250/1	64	73	
PMF1	Suspicion	118/1	65	95	[58, 62]
	Previous history of BC				
Myopodin	Suspicion	164/1	65	80	[57]
	Previous history of BC				
IRF8, p14, and sFRP1	Suspicion	45/1	85	95	[63]
	Previous history of BC	4/1	100	—[c]	
MYO3A, CA10, NKX6-2, DBC1, and SOX11 or PENK	Suspicion	198/1	85	95	[64]
	Previous history of BC	40/1	85	—	
ZNF154, EOMES, HOXA9, POU4F2, TWIST1, and VIM	Suspicion	79/1	98	100	[38, 65]
	Previous history of BC	206/1	94	66	
TWIST1 and NID2	Suspicion	278/1	90	93	[66]
	Previous history of BC				
RASSF1A, E-cadherin, APC, DAPK, MGMT, BCL2, h-TERT, EDNRB, WIF1, TNFRSF25, and IGFBP3	Suspicion				[79]
	Previous history of BC	40/1	86	8	
APC, RASFF1A, RARB, DBC1, SFRP1, SFRP2, SFRP4, SFRP5	Suspicion	146/1	52	100	[67]
	Previous history of BC				
SFRP1, SFRP2, SFRP4, SFRP5, VIF-1, and DKK3	Suspicion	264/1	61.1	93.3	[50, 68]
	Previous history of BC				
RASSF1a, E-cad, and APC	Suspicion	104/1	69	60	[39, 69]
	Previous history of BC				
APC_a, TERT_a, TERT_b, and EDNRB	Suspicion				[80]
	Previous history of BC	94	72	55	

[a]Only studies that include patients with a suspicion of bladder cancer.
[b]Only studies that include patients with a previous history of bladder cancer.
[c]Specificity in studies using healthy individuals as controls is not shown.

a laboratory and requires a large number of exfoliated cells [21]. Applying a cut-off point of at least one green or one red fluorescent cell, the test sensitivity and specificity of the ImmunoCyt assay with 2,896 participating patients in eight studies are 84% (77–91; 95% CI) and 75% (68–83; 95% CI), respectively (Table 1). The test sensitivity for low-risk tumors is 81%, with a range from 55% to 90%, whereas high-risk tumors have a test sensitivity of 90%, with a range from 67% to 100% [12]. In patients with a history of NMIBC, the test sensitivity is 81% and the specificity is 75% (Table 2) [12].

3.3. UroVysion—Fluorescence In Situ Hybridization (FISH). UroVysion is a multitarget fluorescence in situ hybridization (FISH) assay that utilizes DNA probes to identify aneuploidy in chromosomes 3, 7, 17, and loss of the 9p21 locus of the P16 tumor suppressor gene. The assay requires exfoliated cells in the urine. The probe binds to the complementary DNA in these cells, thereby visualizing the location of the targeted chromosomes. The recommended cutoff for a positive result for bladder cancer is usually defined as finding more than five urothelial cells with a gain of more than two chromosomes or ten cells with a gain of a single chromosome, or 12 or more cells with homozygous loss of the 9p21 locus. However, the cutoff may vary between institutions performing this assay. Applying this cutoff or a cutoff very close to this, the sensitivity and specificity of the ImmunoCyt assay with 2535 participating patients in 12 studies are 76% (65–84; 95% CI) and 85% (78–92; 95% CI), respectively (Table 1). The test sensitivity of low-risk tumors is 65%, with a range from 32% to 100%, whereas high-risk tumors have a test sensitivity of 95%, with a range from 50% to 100% [12]. In patients with a history of NMIBC, the test sensitivity is 64% and the specificity is 73% (Table 2) [12].

4. Investigational Bladder Cancer Markers

Presently, there are several investigational urinary biomarkers of bladder cancer which are not yet commercially available, but the most promising will perhaps be approved as urinary markers in the future. A noteworthy group of these promising emerging markers is DNA methylation markers. These markers have some advantages that make them promising as tumor markers: (i) the DNA is quite stable; (ii) methylation can be detected by sensitive real-time PCR assays in a high through-put manner; (iii) results are not dependent on subjective analysis. The remainder of this paper will focus on DNA methylation markers and their application for diagnosis and surveillance in bladder cancer.

4.1. DNA Methylation in Cancer. Cancer initiation and progression are driven by the accumulation of inherited or acquired DNA alterations. These changes may be genetic or epigenetic in nature [22]. Epigenetic changes are defined as heritable changes in gene function which do not involve changes in DNA sequence. Although DNA methylation and histone modifications (methylation, phosphorylation, acetylation) are all epigenetic modifications, only DNA methylation is included in the review. DNA methylation is, almost exclusively, the attachment of a methyl group to the 5th carbon in cytosine positioned just upstream of a guanosine (CpG). Most CpG sites are sparsely distributed throughout the genome and methylated, with the exception of CpG sites located in clusters, termed CpG islands. The majority of CpG islands are unmethylated and located within the promoter regions and exon 1 of more than 50% of all known genes [23–25]. CpG dinucleotides outside CpG islands are generally methylated in normal cells and undergo a substantial loss of DNA methylation in cancers. Increased genetic instability was observed in DNMT1 and DNMT3b double knock-out cells [26] and cells with hypomorphic allele of DNA methyltransferase 1 [27]. Mechanisms linked to hypomethylation induced genetic instability include decondensation of chromatin into recombination permissive conformation [28] and activation of retrotransposon elements [29]. Furthermore, hypomethylation of enhancer sites or other regulatory sites repressed by methylation may trigger increased expression of cancer promoting genes [22, 30, 31]. CpG sites within CpG islands are usually in an unmethylated state, permissive to transcription in normal cells, but become hypermethylated at certain promoters in cancers. DNA methylation within CpG islands, located in promoter regions, is involved in the silencing of DNA transcription together with histone modifications [32]. Development, genomic imprinting, and X-chromosome inactivation are critical normal processes in which DNA methylation occurs [33–35]. Alterations in epigenetic control have been associated with several human pathologic conditions including cancer [36].

4.2. DNA Methylation and Bladder Cancer: Tumor Tissue. With the use of microarray technology, the number of genes reported to be aberrantly methylated in bladder cancer tissue is now in the thousands [37, 38]. It has been shown that high-risk tumors, generally, have more hypermethylated genes than do low-risk tumors [37, 38]. Wolff and colleagues suggest two different epigenetic pathways depending on whether the tumor is muscle-invasive or not [37]. An important finding for the use of methylation as a tumor marker comes from a study with 10 patients. A study of metachronous tumors from these 10 patients indicated that the change in tumor methylation from normal urothelium was stable within patients [38]. Multiple studies by separate groups present data that aberrant methylation is associated with the stage and grade of the tumors, as well as the recurrence rate and risk of progression [38–48]. Transcriptional inactivation by CpG island promoter hypermethylation is a well-established mechanism for gene silencing in cancer including bladder cancer [46, 49–58]. Many genes have been reported aberrantly methylated in bladder cancer, but relatively few of the genes have been characterized with respect to the function of the attendant gene silencing.

Age-dependent methylation of CpG islands occurs in a tissue-specific manner in normal appearing tissue [59, 60], and in bladder cancer increased methylation of CpG islands has been reported to be associated with high age of the patients [44, 61]. Examples of CpG islands in which methylation is associated with high age include CCNA1, PGP9, and CCND2 [44]. These findings make it of paramount importance to include age-matched controls when identifying diagnostic DNA methylation markers for bladder cancer.

5. DNA Methylation and Bladder Cancer: Urine

5.1. Diagnosis of Urothelial Carcinoma of the Bladder. Several studies have reported results indicating that methylation markers, applied to DNA from voided urine or bladder washes, can be used for the diagnosis of bladder cancer (Table 3). Nine studies presented results from patients with

Table 3: Performance of DNA methylation markers in studies that include patients with suspicion of bladder cancer and patients previously diagnosed with bladder cancer.

Test	Method	Patients[a]/ studies	Sensitivity, %	Specificity, %	Interference by other bladder conditions	Reference
PMF1	MSP	118/1	65	95	No	[58, 62]
Myopodin	MSP	164/1	65	80	No	[57]
RASSF1A	MSP	24/1	50	100	Not done	[70]
DAPK, RARβ, E-cadherin, and p16	MSP	39/1	91	76	Not done	[71]
IRF8, p14, and sFRP1	qMSP	49/1	87	95	No information	[63]
MYO3A, CA10, NKX6-2, DBC1, and SOX11 or PENK	qMSP	238/1	85	95	No	[64]
GDF15, TMEFF2, and VIM	qMSP	110/1	94	90	Not done	[72]
APC, RASSF1A, and $p14^{ARF}$	MSP	66/1	87	100	No	[73]
DAPK, BCL2, and TERT	qMSP	57/1	78	100	Not done	[42, 74]
CDKN2A, ARF, MGMT, and GSTP1	qMSP	269/1	69	100	No	[41, 75]
RASSF1A, p14, and E-cadherin	MSP	66/1	80	100	Not done	[76]
ZNF154, HOXA9, POU4F2, and EOMES	MS-HRM	174/1	84	96	No	[38, 65]
TWIST1 and NID2	qMSP	278/1	90	93	No	[66]
APC, RASFF1A, RARB, DBC1, SFRP1, SFRP2, SFRP4, SFRP5	qMSP	146/1	52	100	No	[67]
SFRP1, SFRP2, SFRP4, SFRP5, VIF-1, and DKK3	MSP	264/1	61.1	93.3	Not done	[50, 68]
BCL2 and hTERT	qMSP	213/1	76	98	No	[21]
RASSF1a, E-cad, and APC	qMSP	104/1	69	60	No	[39, 69]
SALL3, CFTR, ABCC6, HPR1, RASSF1A, MT1A, RUNX3, ITGA4, BCL2, ALX4, MYOD1, DRM, CDH13, BMP3B, CCNA1, RPRM, MINT1, and BRCA1	MSP	159/1	92	88	No	[77]

[a] Including both patients with BC and individuals with no history of bladder cancer.
[b] Not applicable.

a suspicion of bladder cancer [57, 62–69], whereas other studies included a mixture of samples from patients with a suspicion of bladder cancer, and patients previously diagnosed with bladder cancer, or the information was not reported [21, 38, 70–77]. Patients with a confirmed suspicion of bladder cancer will generally have larger tumors that shed more cells into the urine than patients under surveillance for bladder cancer. Cancer cells shed from tumors within the bladder may be of low or high grade. Low-grade tumors are less likely to shed many cells into the urine as their high-grade counterparts because the high-grade tumors have weaker intercellular attachments [78]. Depending on the detection method this may influence the sensitivity of the urinary markers.

The large variation of cell types in the urine necessitates that the DNA methylation markers are cancer specific and uninfluenced by hematuria, bladder infections, or other benign bladder conditions. Many of the studies have included individuals with a mixture of benign urothelial conditions to insure the cancer specificity of the methylation markers, although some studies have either not done this or were not reported.

The first study demonstrating the feasibility of diagnosing bladder cancer through methylation using DNA from voided urine came from Chan and colleagues in 2002 [71]. They analyzed urine sediments from voided urine from 22 patients and 17 age- and sex-matched controls by methylation sensitive PCR (MSP). The analysis was based on a panel of markers (DAPK, RARβ, E-cadherin, and p16) that achieved a sensitivity of 91% and a specificity of 76% (Table 3) [71]. In the same study, the sensitivity and specificity of cytology were 46% and 100%, respectively. Subsequently, many studies have reported methylation markers with an increased sensitivity, but a lower specificity than cytology. Investigations of matched tumor and urine samples showed no false positive urine samples when the tumor was negative for methylation, indicating the specificity of the markers. Unfortunately, Chan et al. did not provide any information about bladder conditions within the control group.

The fact that DNA methylation markers may be as specific as cytology was revealed in a study by Dulaimi et al. with 45 bladder cancer patients and 21 healthy age-matched individuals including 9 individuals with cystitis. The sensitivity was only 87%, but the specificity was 100% (Table 3).

The concordance between methylation in the tumor and the matched urine was very high, and no urine sample was positive without simultaneous tumor methylation [73].

Quantitative real-time PCR was introduced by Friedrich et al., who investigated 37 bladder cancer patients undergoing radical cystectomy, 10 age-matched cancer-free individuals, and 10 non-age-matched cancer-free individuals. A panel of markers (DAPK, BCL2, and TERT) achieved a sensitivity of 78% and a specificity of 100% (Table 3) [74]. Currently, real-time PCR is the most applied technique, owing to the low DNA requirement and high sensitivity, compared with MSP.

A different technique was applied by Reinert and coworkers when they used methylation-sensitive high-resolution melting (MS-HRM) PCR to study voided urine from 119 bladder cancer patients and 59 individuals with no history of bladder cancer. The controls were from patients with benign prostatic hyperplasia (BPH) or bladder stones, and 19 patients were suspected of having a bacterial infection. In that study, a four-marker panel (ZNF154, HOXA9, POU4F2, and EOMES) achieved a sensitivity of 84% and a specificity of 96% (Table 3) [38]. Recently, a validation of ZNF154, HOXA9, POU4F2, and EOMES by real-time PCR (MethyLight) using DNA from 184 bladder cancer patients and 35 control individuals resulted in an increased sensitivity of the individual markers by eight to twenty-five percentage points (Table 2) [38, 65]. The increased test sensitivity is probably due to more sensitive MethyLight primers, as the patient cohorts are comparable.

Notably, a prospective multicenter study with both training and validation sets with a total of 83 bladder cancer patients and 178 individuals with no history of bladder cancer achieved a sensitivity of 90% and a specificity of 93%. The control patients were all diagnosed with benign urologic diseases (Table 2) [66].

Several additional studies have confirmed that urinary markers may be highly sensitive for the detection of bladder cancer and at the same time very specific, even when examining patients with other urological diseases (Table 3). Lately, numerous methylation markers identified by screening of bladder cancer tissue samples or cell lines show high sensitivity and specificity when translated to BC detection in urine [38, 64, 66, 72, 77]. Bladder marker studies require age-matched controls to ensure that the observed methylation is tumor specific, since methylation within CpG islands may increase with age in a tissue-specific manner [60]. Most studies have included age-matched controls, but in the study by Lin et al. the median age between bladder cancer patients and healthy individuals varied by more than 30 years [76]. Many studies have reported a high concordance between tumor tissue and urine specimen methylation, indicating that the methylation detected was specific for bladder cancer cells and did not reflect methylation of DNA from other sources in the urinary system [21, 38, 67, 70–73, 75–77, 81]. Deviating from this was Vinci et al. who found the methylation status of DAPK to be discordant between tumor and urine sediments [21]. Several methylation markers have shown higher sensitivity with increasing stage [21, 38, 68, 75, 81] and grade [21, 38, 65, 70, 81]. Two studies have reported that the methylation markers reported in their studies were not independent of each other [38, 75]. This indicates that one single methylation mechanism may account for the majority of the methylation alterations analysed in those studies.

Methylation markers for bladder cancer diagnosis are still at an early stage compared with the FDA approved markers. Most of the reported markers have been tested on cohorts that varied greatly between studies. In addition to this, many markers are lacking validation in independent prognostic experiments with predetermined cut-off values. Independent validation experiments will often achieve lower test sensitivity and/or lower test specificity, as the cut-off values from the initial experiment were fitted to the data. With that said, comparing the methylation markers with cytology, it is evident that the methylation markers are more sensitive than cytology, and for the some of the markers the specificity is even comparable with cytology (Table 2). Nevertheless, methylation markers have not entered the clinical setting as diagnostic markers of bladder cancer instead of cystoscopy or as a supplement to cystoscopy or cytology.

5.2. Urinary DNA Methylation Markers for Surveillance of Bladder Cancer. Patients previously diagnosed with bladder cancer are included in a treatment regimen with frequent follow-up cystoscopy examinations, and perhaps cytology as an adjunct. Cystoscopy is an invasive procedure that is costly for the healthcare system and unpleasant for the patient. The search for a noninvasive DNA methylation marker for the diagnosis of bladder cancer was initiated more than a decade ago by Chan and coworkers [71], whereas the search for a diagnostic DNA methylation marker for surveillance of bladder cancer patients only started in 2008 with a study by Rouprêt et al. [79]. They designed a control group from bladder cancer patients with a negative cystoscopy and/or biopsies. In this small study with 40 patients of which 15 experienced a recurrence, they reported the sensitivity of an 11 gene panel to be 86%, while the specificity was as low as 8% (Table 2). In a more recent study by Zuiverloon et al. they developed a four gene markers panel by screening NMIBC tissue, urine from nonbladder cancer patients, and blood from bladder cancer patients for methylation of 37 genes. In the validation cohort with 94 bladder cancer patients under surveillance, the sensitivity of this panel of urinary markers was 72% and the specificity was 55% (Table 2) [80]. In a most recent and yet unpublished study by Reinert et al. [65], they tested six methylation markers that have previously been shown to be able to differentiate between bladder cancer patients and individuals with no history of bladder cancer with high sensitivity and specificity [38]. When including only urine samples from patients that had previously tested positive for methylation, and analyzing 206 voided urine samples from patients under surveillance for bladder cancer, they achieved a test sensitivity of 93% and a specificity of 47% of a single marker when comparing to cystoscopy carried out at the same visit to the clinic. Including cystoscopy results during a 12-month follow-up period in the analysis, they found many of the samples formerly classified as false positives to be true positives (anticipatory positives).

The adjusted methylation marker sensitivity and specificity were 94% and 66%, respectively. Importantly, in these settings all six markers were independent of stage and grade.

Anticipatory positives are results that show a positive finding preceding visual evidence of a bladder tumor. The term "anticipatory positive" is debatable, due to the high recurrence rate in bladder cancer. However, a prognostic study with 250 recurrent cases by Yoder and colleagues focusing on anticipatory findings showed that of 148 patients with negative cystoscopy, 5% of UroVysion negative and 65% of UroVysion positive results experienced a recurrent urinary cancer within 29 months after cystoscopy [82]. In a similar manner, the anticipatory effect has been shown for DNA methylation markers by Reinert and colleagues, who found that 4% of methylation-negative and 63% of methylation-positive patients relapse within 12 months [65].

5.3. Urinary DNA Methylation Markers for Prediction of Recurrence Free Survival. While several studies have focused on diagnostic markers and markers of surveillance, the potential of urinary methylation markers as prognostic markers of recurrence has just recently been suggested in a single and yet unpublished study by Reinert et al. [65]. They reported that detection of DNA methylation of ZNF154, HOXA9, POU4F2, TWIST1, or VIM was significantly associated with future recurrences, with hazard ratios ranging from 7.8 to 13.9. They speculated that the prognostic value of the methylation markers was connected with the existence of a DNA hypermethylation field disease as reported previously [37].

6. Conclusion

Early diagnosis of bladder cancer, and careful followup for detection of recurrences after initial treatment, are main tasks of current urological research. The high rate of recurrences and the prolonged followup by cystoscopy and cytology make bladder cancer the most expensive cancer to treat, overall. However, by utilizing noninvasive urinary markers, it may be possible to improve the diagnosis of new cancers as well as improve the management of NMIBC. NMP22, UroVysion, and ImmunoCyt are well-examined urinary markers. NMP22 and UroVysion are both FDA-approved tests for initial diagnosis of bladder cancer in patients with a suspicion of bladder cancer and for surveillance of bladder cancer; however, ImmunoCyt is only approved for the surveillance of bladder cancer in conjunction with urinary cytology and cystoscopy. All three markers have a higher sensitivity than cytology, but a lower specificity (Table 1). Furthermore, the specificity of NMP22 and ImmunoCyt are influenced by urinary conditions other than bladder cancer, which makes them unusable in many situations (Table 1).

The methylation markers are not as well studied as the FDA-approved markers, and none have been approved by the FDA for the diagnosis or surveillance of bladder cancer. Weaknesses of the methylation studies include (1) many small studies with a limited number of participants; (2) different methods for analyzing methylation between studies making comparison of markers difficult; (3) low homogeneity of study population regarding stage and grade of the patients; (4) lack of information about adjuvant intravesical therapy; (5) heterogeneous patients populations, as many studies include patients under suspicion for bladder cancer as well as patients with a history of bladder cancer; (6) absence of age- and sex-matched reference groups; (7) retrospectively collected samples that may be biased regarding availability of material for analysis. With these reservations in mind, most methylation markers show higher sensitivity than cytology, but often at the cost of a lower specificity (Table 2). For diagnosis of bladder cancer in patients under suspicion of bladder cancer, the most sensitive and specific DNA methylation markers are probably preferable compared with the FDA-approved markers, owing to the higher sensitivity and specificity of the methylation marker, in addition to the fact that most of the DNA methylation markers have been shown to be uninfluenced by other benign bladder conditions or benign prostatic hyperplasia.

For surveillance of bladder cancer patients, DNA methylation markers have the highest sensitivity (94%) followed by ImmunoCyt (81%), NMP22 (69%), UroVysion (64%), and cytology (38%). Cytology has the highest specificity (94%) followed by NMP22 (81%), ImmunoCyt (75%), UroVysion (73%), and DNA methylation markers (66%). In situations where high test sensitivity is important, DNA methylation markers ought to be the test of choice, whereas if a high specificity is important, cytology should be the preferred marker. Considering the question whether or not a urinary marker may postpone the time between cystoscopies or replace cytology in surveillance of NMIBC, the answer must be that some of the methylation markers are more than capable, because the DNA methylation markers have higher sensitivity than white light cystoscopy and cytology. This assumes that the current results can be validated in independent prognostic studies. In addition, it will be important to have some comparison studies in which the DNA methylation markers are compared with established markers using standardized assays and cut-off values. In a study by Yossepowitch et al. they conclude that 75% of patients under surveillance for bladder cancer would accept the result of a urine marker test as a replacement for cystoscopy, if the sensitivity of the marker was at least 95% [83]. It is worth considering that the patients interviewed were expecting the sensitivity of cystoscopy to be 100%, which is not true in all cases. A suggested surveillance regime for low- and medium-risk NMIBC could be to replace the cystoscopies at 12, 24, and 48 months, after the removal of the tumor, with methylation markers (Figure 1). The potential of the methylation markers is unmatched by cytology and the FDA-approved markers with respect to detecting recurrent tumors. However, until additional prospective studies of suitable proportions have validated the promising results, surveillance will continue to consist of cystoscopy and cytology.

7. Future Perspectives

Detecting bladder cancer using diagnostic or surveillance markers remains a challenge, as none of the currently approved markers can replace cystoscopy or prolong the time between cystoscopies. The field of methylation biomarkers is

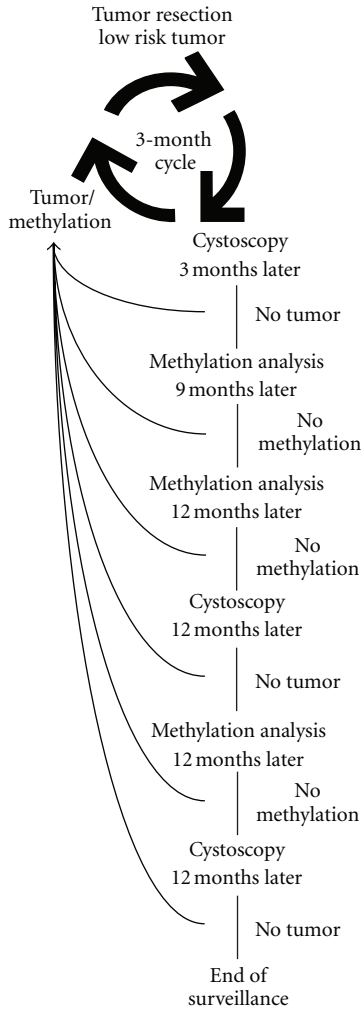

Figure 1: Follow-up model for low- and medium-risk NMIBC in which methylation markers were applied to prolong time between cystoscopies.

promising, but requires more prospective multicenter studies that explicitly clarify the purpose of the study as screening, diagnosis of primary disease, or surveillance. In addition, these studies should include the appropriate patients in order to validate the current findings in an unbiased manner. Currently, at least one such study combining methylation markers and FGFR3 mutational markers for surveillance of bladder cancer is ongoing (UROMOL, FP7 EU study). This study investigates urine samples from more than 1200 patients with bladder cancer in several clinical centers in Europe.

Recent technological advances within the field of methylation analysis, like the Illumina Infinium HumanMethylation450 BeadChip, or some of the many next generation sequencing-based methylation analyses, for example, whole-genome shotgun bisulfite sequencing, may uncover even more sensitive and specific markers in the future. Nevertheless, the emphasis should be to develop existing markers and in parallel identify new promising methylation markers in order to increase survival and the quality of life for bladder cancer patients.

Conflict of Interests

The author declares that he has no conflict of interests.

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