BLADDER CANCER – A CINDERELLA CANCER: ADVANCES AND REMAINING RESEARCH QUESTIONS

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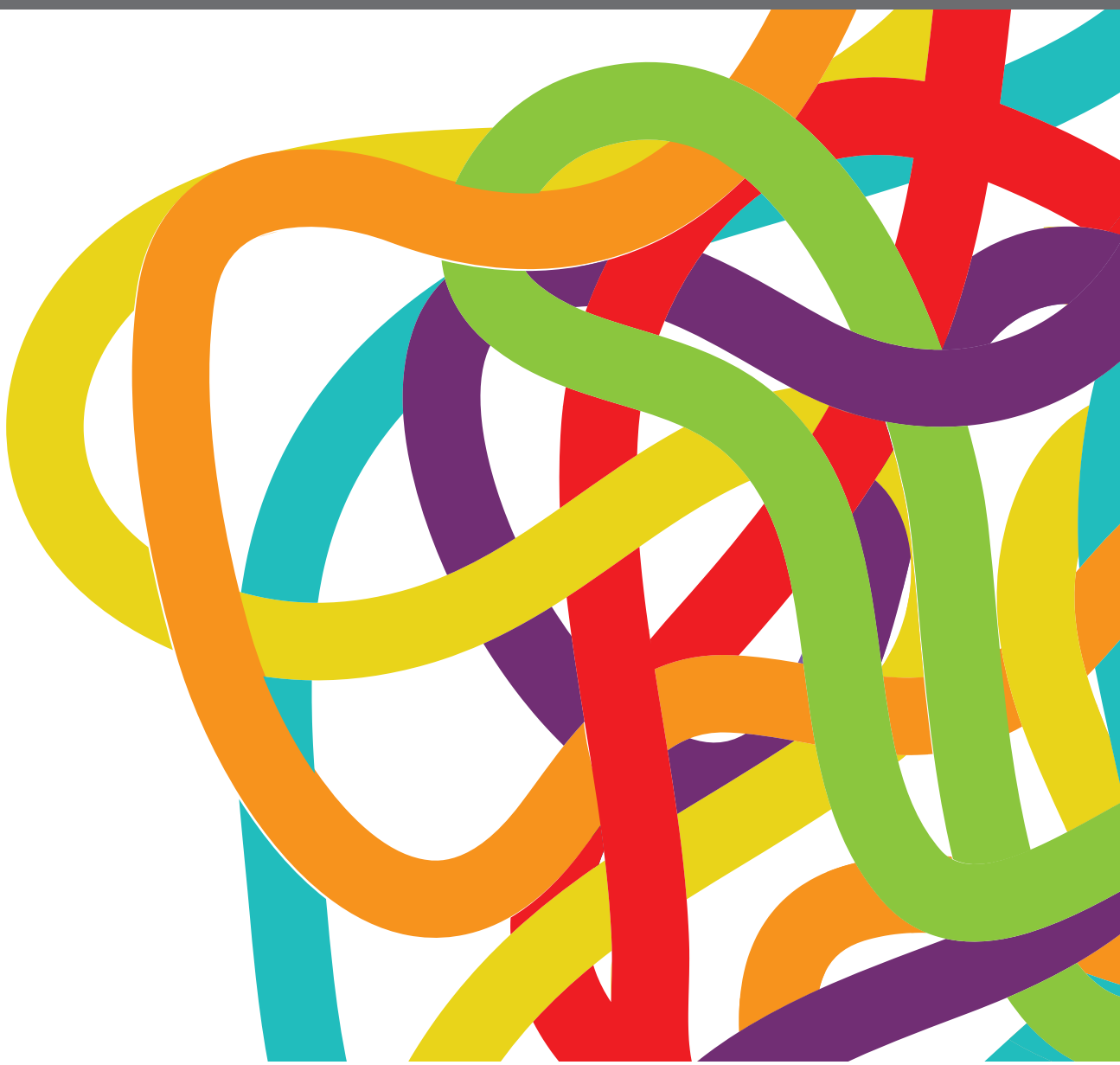

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BLADDER CANCER – A CINDERELLA CANCER: ADVANCES AND REMAINING RESEARCH QUESTIONS

Topic Editors:
Mieke Van Hemelrijck, King's College London, United Kingdom
Prashant Patel, University of Birmingham, United Kingdom
Kent William Mouw, Dana–Farber Cancer Institute, United States

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EDITORIAL
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Editorial: Bladder Cancer – A Cinderella Cancer: Advances and Remaining Research Questions

Mieke Van Hemelrijck[1]*, *Prashant Patel*[2] and *Kent W. Mouw*[3]

[1] School of Cancer and Pharmaceutical Sciences, Translational Oncology and Urology Research (TOUR), King's College London, London, United Kingdom, [2] Institute of Cancer and Genomic Sciences, University of Birmingham, Birmingham, United Kingdom, [3] Department of Radiation Oncology, Dana–Farber Cancer Institute/Brigham & Women's Hospital and Harvard Medical School, Boston, MA, United States

Keywords: bladder cancer, diagnostics, personalization, surveillance, prognostic

Editorial on the Research Topic

Bladder Cancer – A Cinderella Cancer: Advances and Remaining Research Questions

Bladder cancer is the 4th most common male cancer and 9th most common female malignancy. Despite its high incidence and prevalence, clinical outcomes have been largely static over the past 25 years. In 2010, bladder cancer was the 9th most expensive cancer in the USA with cumulative costs of 4 billion US dollars or 3.2% of all cancer-related care. A potential and significant contributing factor for the relative lack of improvement in the static mortality rate of BC is the small investment in bladder cancer research. In the UK, prostate cancer research is supported with over £26,458,355 in funding and £561 are spent per new patient. However, research spent on bladder cancer was only £3,886,966 with £382 spent per new patient. A similar funding discrepancy is seen in the USA. In addition to the lack of research funding, bladder cancer has the highest lifetime treatment costs per patient of all cancers. This historical lack of funding means there are now many important unanswered research questions, making prioritization very challenging (1).

Our understanding of factors influencing BC risk and development is improving, but variability across ethnicities has been observed. Wu et al. performed a three-stage case-control study including 3,399 BC patients and identified a novel rare coding variant that increased BC risk in Han Chinese. This may inform future development of novel targeted agents in BC development and progression.

However, it is important to note that the diagnostic pathway of BC is not straightforward due to the high potential of delayed presentation and misinterpretation of potential symptoms like haematuria. Nevertheless, urine biopsies have been suggested to be "liquid gold" as they are less invasive than tumor tissue biopsies and can be an abundant source of tumor-derived material (Satyal et al.). The molecular detection of mutations in urine DNA requires a sensitive and accurate method of analysis—so despite its potential for diagnosis, prognosis, and monitoring of tumor evaluation, significant challenges are apparent and research is still evolving. It is therefore exciting to note that Lipunova et al. externally replicated an algorithm for BC prognosis based on urinary polymorphisms using data from the UK Biobank.

Many other initiatives are ongoing in the area of BC risk stratification to evaluate and refine both new markers as well as existing procedures, such as performing restaging transurethral resection (i.e., repeat TUR) (Calò et al.) and evaluating pre-treatment neutrophil-lymphocyte ratios (Suh et al.). In addition, specific gene expression profiles have been associated with poor prognosis in patients with non-muscle invasive BC (NMIBC) (Chen et al.). By investigating the relation between circular RNAs (circRNAs) and tumor grade in a cohort of NMIBC, another study showed that the assessment of expression levels of circRNAs may provide an additional layer of information for patient stratification (Goel et al.). Apart from providing new avenues for precision medicine, these

Edited and reviewed by:
Ronald M. Bukowski, Cleveland Clinic, United States

***Correspondence:**
Mieke Van Hemelrijck
mieke.vanhemelrijck@kcl.ac.uk

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studies also help us understand how we can bring various observations together—including the rapidly emerging field of immunotherapy and surveillance for BC (Joseph and Enting).

This rapid expansion of diagnostic and prognostic factors for BC also requires strategic thinking for the management of big data. Zhang et al. developed an online consensus Survival tool for bladder cancer (OSblca) to analyze the prognostic value of specific gene expression patterns by integrating genetic and clinical data from 1,075 BC patients. Another initiative providing opportunities for real world evidence research is data harmonization across various hospitals to provide a prospective database with detailed baseline information as well as clinical follow-up—such a database for NMIBC was set-up in Belgium in 2013 (Akand et al.).

Finally, whilst it is encouraging to see a significant increase in BC research, all researchers should be encouraged to engage with BC patients and their carers when designing and conducting research studies as to ensure the best impact on their care (MacLennan and MacLennan).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Is Repeat Transurethral Resection Always Needed in High-Grade T1 Bladder Cancer?

*Beppe Calò[1], Marco Chirico[1], Francesca Fortunato[2], Francesca Sanguedolce[3], Emanuel Carvalho-Dias[4], Riccardo Autorino[5], Giuseppe Carrieri[1] and Luigi Cormio[1]**

[1] Urology and Renal Transplantation Unit, Department of Medical and Surgical Sciences, University of Foggia, Foggia, Italy, [2] Department of Epidemiology, University of Foggia, Foggia, Italy, [3] Section of Pathology, Department of Clinical and Experimental Medicine, University Hospital of Foggia, Foggia, Italy, [4] Urology Department, Hospital de Braga ICVS, University of Minho, Braga, Portugal, [5] Division of Urology, Department of Surgery, VCU Health, Richmond, VA, United States

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Edited by:
Prashant Patel, University of Birmingham, United Kingdom

Reviewed by:
John Parkin, Sandwell & West Birmingham Hospitals NHS Trust, United Kingdom
Ari Adamy, Santa Casa Hospital, Brazil

***Correspondence:**
Luigi Cormio
luigi.cormio@unifg.it

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Re-staging transurethral resection, the so-called repeat TUR (Re-TUR), is mandatory in case of incomplete first transurethral resection of bladder tumor (TURBT). In completely resected high grade T1 tumors, Re-TUR is recommended but question remains whether it provides advantages in terms of recurrence-free survival (RFS), progression-free survival (PFS), and cancer specific survival (CSS). The present study aimed to determine whether Re-TUR improves such outcomes in patients with completely resected high-grade T1 bladder cancer (BC). We queried our prospectively maintained database to identify patients with completely resected high-grade T1 BC who underwent (Group A) or not (Group B) Re-TUR before starting intravesical instillations of Bacillus Calmette-Guerin (BCG). The impact of Re-TUR as well as of other tested variables on RFS, PFS, and CSS was tested by Kaplan-Meier method and Log-rank testing. A total of 118 patients underwent Re-TUR, which pointed out no BC in 61 (51.7%), NMIBC in 54 (45.8%) and pT2 disease in 3 (2.5%). The 3 patients with pT2 disease underwent cystectomy, whereas all others were offered BCG treatment. Forty-two patients refused BCG treatment while 2 did not complete it; therefore, Group A (Re-TUR before BCG treatment) consisted of 71 patients whereas Group B consisted of 40 patients who refused Re-TUR but completed BCG treatment. Mean follow-up was 60 months (range 12-142). Kaplan-Meier curves and Log-rank testing showed no difference in RFS, PFS and CSS between patients who had (Group A) or had not (Group B) Re-TUR before starting BCG treatment. Our findings suggest that a Re-TUR in patients with a completely resected high-grade T1 BC does not translate into a better oncological outcome. Given its impact on both patients and healthcare system, the need for Re-TUR in completely resected high grade T1 BC should be further investigated into the framework of a randomized study.

Keywords: non-muscle-invasive bladder cancer, second transurethral resection, Bacillus Calmette-Guerin, T1, high-grade

INTRODUCTION

The first and mainstay approach in the diagnosis and treatment of bladder cancer (BC) is transurethral resection of the bladder tumor (TURBT). Complete resection is crucial in the management of patients with high-risk non-muscle-invasive bladder cancer (NMIBC) (1). A restaging transurethral resection, the so-called repeat TUR (Re-TUR), which is mandatory in case of incomplete first resection, is currently recommended in high-grade T1 tumors, either primary or recurrent, even in case of a complete first TURBT (2).

In one of his first study about this issue Herr claimed that Re-TUR was especially appropriate for patients with T1 tumors to confirm complete resection and to detect muscle invasion (3). In the following years it has been shown that this can provide additional pathologic information, as residual Ta/T1 lesions are found in 33–55% of patients and muscle-invasive (T2) disease can be detected in 10–25% of patients (4–7). Question however remains whether Re-TUR provides advantages in terms of recurrence-free survival (RFS), progression-free survival (PFS), and cancer specific survival (CSS).

The first and only randomized controlled trial (RCT) addressing this issue (8) pointed out that, in patients with T1 BC, Re-TUR provided a significant benefit in RFS and PFS but not in CSS. It is worth mentioning that almost half of patients had a T1 low-grade disease and that patients received intravesical instillations of Mitomycin-C rather than of Bacillus Calmette-Guerin (BCG), which should be the standard treatment for high-risk NMIBC (8, 9). The largest retrospective study testing the role of Re-TUR in a homogeneous population of patients with high-grade T1 BC treated with intravesical BCG (10) conversely pointed out that, in case of complete resection, Re-TUR did not improve RFS, PFS, and CSS. These findings would question the need for such additional surgical procedure, considering its patients and healthcare burdens. The present study aimed to determine the effect of Re-TUR on RFS, PFS, and CSS in a homogeneous population of patients with high-grade T1 BC treated with BCG.

MATERIALS AND METHODS

Our prospectively maintained NMIBC database was queried to identify patients with high-grade T1 BC, either primary or recurrent, who underwent Re-TUR (Group A) or did not undergo Re-TUR (Group B) before receiving adjuvant intravesical instillations of BCG. The study was approved by the Internal Review Board, Nephro-Urological Department, Foggia University Hospital, Foggia, Italy. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Study inclusion criteria were: (i) complete resection, i.e., no visible tumor left behind and bladder muscle clearly identifiable by pathologist and free of disease; (ii) having completed the BCG treatment (induction by one instillation a week for 6 consecutive weeks and maintenance by one instillation a month for 12 months); (iii) having undergone bladder biopsies (random and/or visible lesions) 4–8 weeks after having completed the BCG induction cycle. Patients with incomplete clinical data were excluded.

Re-TUR involved resection of any visible tumor, deep resection of previously resected areas, and random bladder biopsies. As mentioned above, all patients underwent urine cytology and bladder biopsies (random and/or visible lesions) 4–8 weeks after having completed the BCG induction cycle, as we aimed to evaluate the role of this diagnostic pathway in assessing the results of BCG treatment.

Follow-up consisted of urine cytology and cystoscopy every 3 months for the first two years, every 6 months for the third year, and then yearly. A CT urogram was also performed at initial diagnosis and then every year to rule out upper tract or metastatic disease. Tumor recurrence was defined as pathological evidence of disease at bladder biopsy or TURBT, whereas tumor progression was defined as pathological shift to muscle invasive disease at bladder biopsy or TURBT or evidence of metastatic disease.

Two senior pathologists unaware of clinical data reviewed all specimens including agreement with the latest WHO Classification of Tumors of the Urinary System and Male Genital Organs (11) and the 2010 TNM staging system (12). The study was approved by the Internal Review Board.

Statistical Analysis

Continuous data are reported as means ± standard deviations (SD) or median values as appropriate.

Those with normal distribution according to the Skewness and Kurtosis test were compared by Student's *t*-test for paired or unpaired data, whereas those with a non-parametric distribution were compared by the Mann-Whitney *U*-test. Differences in rates were compared by the chi-square test or the Fisher's exact test. Univariate survival analysis was carried out using the Kaplan-Meier method, with differences among groups being tested for significance using the Log-rank test. Significance was set at $p < 0.05$. Statistical analysis was carried out using the MedCalc 16.8 Software (MedCalc, Ostend, Belgium) and STATA SE 14.

RESULTS

From January 2005 to January 2017, a total of 184 patients with high-grade T1 BC were offered Re-TUR before being scheduled for BCG treatment (**Figure 1**). Forty patients refused Re-TUR but completed BCG treatment; they represent controls (Group B). Twenty-six patients were excluded from this study as Re-TUR was carried out because of incomplete resection or absence of detrusor muscle in the specimen. In the remaining 118 patients, Re-TUR (**Table 1**) pointed out no BC in 61 (51.7%), NMIBC in 54 (45.8%), and pT2 disease in 3 (2.5%). The three patients with pT2 disease underwent cystectomy, whereas all others were offered BCG treatment; forty-two patients refused it while two did not complete it, thus leaving 71 patients who had Re-TUR and completed BCG treatment (Group A).

Table 2 compares the characteristics of patients in Group A and B showing that, apart from tumor multifocality being significantly more common in Group A, the two groups had similar characteristics.

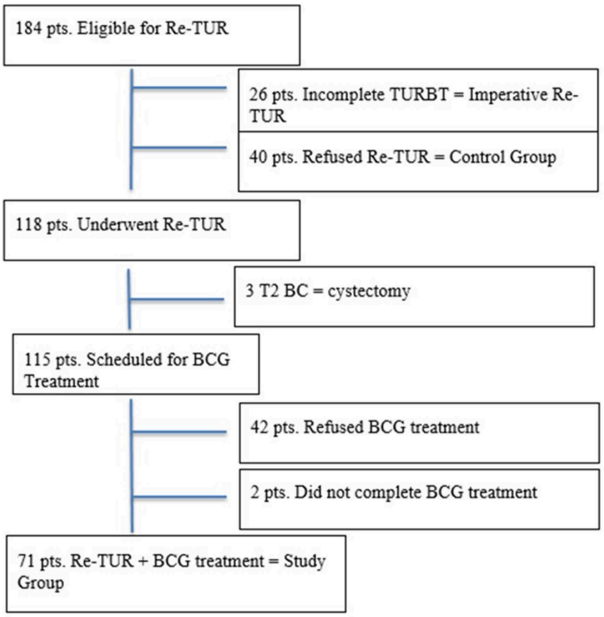

FIGURE 1 | Flowchart patients selection.

TABLE 1 | Results of Re-TUR in completely resected high grade T1 BC.

T2, n (%)	3 (2.5)
T1 HG, n (%)	19 (16.1)
Ta HG, n (%)	10 (8.5)
Ta HG + CIS, n (%)	1 (0.8)
Ta LG, n (%)	7 (5.9)
CIS, n (%)	17 (14.4)
No BC	61 (51.7)

HG, high-grade; LG, low grade; CIS, Carcinoma in situ.

TABLE 2 | Patients' characteristics.

	Group A = 71 pts	Group B = 40 pts	*p-value*
Mean Age (y)	67.9 ± 9.6	69.7 ± 11.2	0.2004
Female Gender, *n* (%)	18%	8.1%	0.1172
Primary, *n* (%)	56 (78.9)	26 (65)	0.1866
Multifocal, *n* (%)	51 (71.8)	11 (28.2)	0.0001
Concomitant CIS, *n* (%)	3 (4.2)	1 (2.5)	0.5365
Diameter BC > 30 mm, *n* (%)	27 (38)	15 (37.5)	0.8990

CIS, Carcinoma in situ.

Bladder biopsies after BCG treatment showed was no difference in the tumor recurrence rate between Group A and B (12.6 vs. 15%, respectively, $p = 0.765$). In particular bladder biopsies after BCG treatment showed, in Group A, 2 high-grade T1 with concomitant CIS, 3 de novo CIS, and 4 low grade Ta BC. The two patients with recurrent high-grade T1 and concomitant CIS underwent cystectomy; those with de novo CIS had a second BCG induction cycle while the others had BCG maintenance. In Group B bladder biopsies after BCG treatment showed 1 CIS, 3 high-grade Ta, 1 high-grade T1, and 1 high-grade T2 BC. Patients with no tumor recurrence or with recurrent low grade disease were scheduled for BCG maintenance; the patient with T2 disease underwent cystectomy. The remaining patients with recurrent high-grade disease or CIS had a second BCG induction cycle.

TABLE 3 | Oncological outcomes.

	All (108 pts)	Group A (69 pts)	Group B (39 pts)	*p*-value*
Recurrence, *n* (%)	33 (30.5)	19 (27.5)	14 (35.8)	0.346
Progression, *n* (%)	17 (15.7)	9 (13)	8 (20.5)	0.199
Cancer-related death, *n* (%)	9 (8.3)	3 (4.3)	6 (15.3)	0.046

**Chi-square test.*

The mean follow-up of the 108 patients (69 in Group A and 39 in Group B) who remained on "conservative" treatment was 60 months (range 12–142). Recurrence occurred in 33 patients, 19 (27.5%) in Group A, and 14 (35.8%) in Group B. Progression occurred in 17 patients, 9 (13%) in Group A, and 8 (20.5%) in group B. Eight progressions occurred after disease recurrence and consisted of local disease in 5, local disease + liver metastases in 1 and local disease + lung metastases in 2; they were 4 (5.8%) in Group A and 4 (10.3%) in Group B. Nine patients presented direct disease progression (7 local diseases and 2 associated to multiple pulmonary metastases); they were 5 (7.2%) in Group A and 4 (10.3%) in Group B. Of the 17 patients who progressed, 11 underwent cystectomy; 9 patients died because of their BC (**Table 3**).

Kaplan-Meier curves (**Figures 2A–C**) and Log-rank testing showed no difference in RFS, PFS and CSS between patients who had or not Re-TUR. Indeed, also the other tested variables had no impact on RFS, PFS, and CSS, as Kaplan-Meier curves and Log-rank testing showed no difference for males vs. females, primary vs. recurrent tumors, single vs. multiple tumors, tumor size <3 cm vs.>3 m, and presence vs. absence of CIS (**Table 4**).

DISCUSSION

Several findings from our analysis are worth discussing within the framework of current literature.

About 46% of our patients undergoing Re-TUR after a completely resected high-grade T1 BC were found to have NMIBC, while only 2.5% were found to have T2 disease. While the rate on NMIBC is in line with previous studies (1, 5, 6), the rate of T2 disease is lower and consistent with that (2.5%) yielded by bladder biopsies after BCG treatment in those who did not undergo Re-TUR. Taken together, these figures somehow speak for appropriate depth of our initial TURBT.

Even more interesting, bladder biopsies after BCG treatment showed no significant difference in the tumor recurrence rate between group A and B. Since it is reasonable to assume

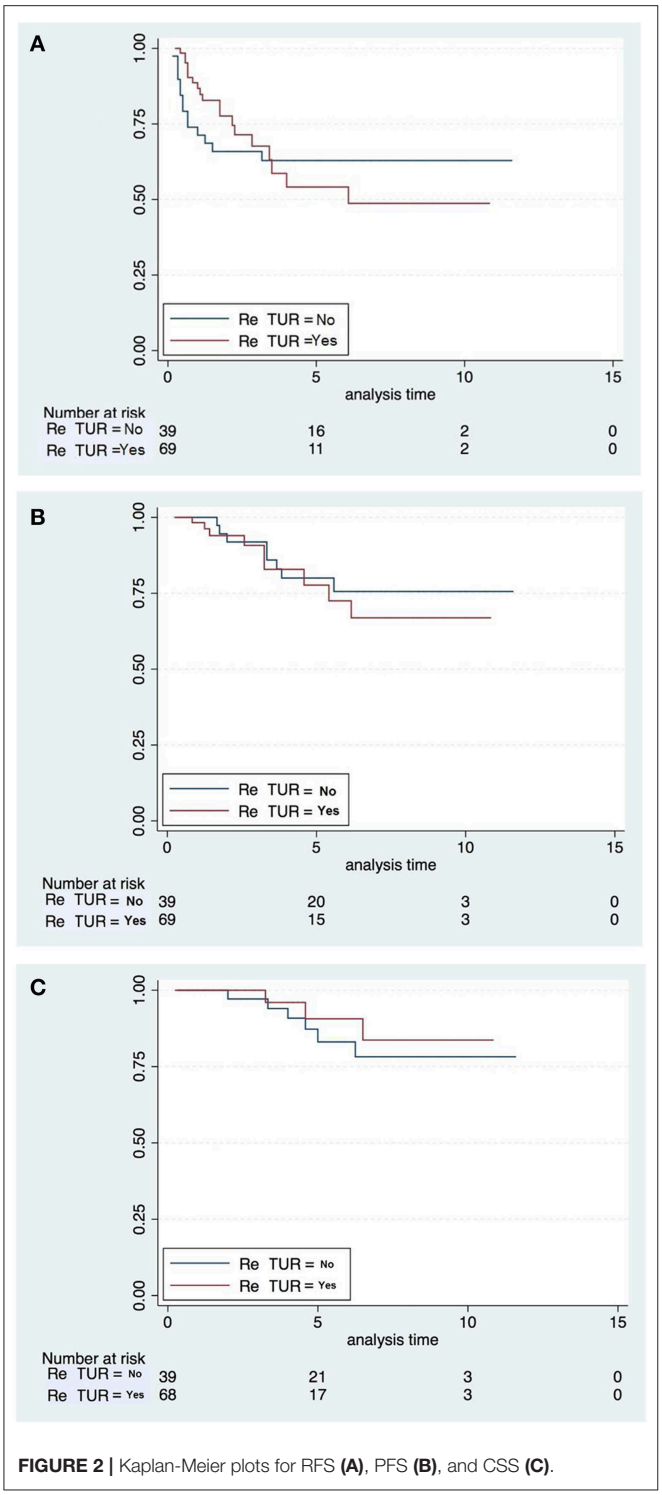

FIGURE 2 | Kaplan-Meier plots for RFS **(A)**, PFS **(B)**, and CSS **(C)**.

TABLE 4 | Univariate analysis* of impact of tested variables on recurrence-free survival (RFS), progression-free survival (PFS) and cancer-specific survival (CSS).

	RFS	PFS	CSS
Male vs. Female	0.137	0.118	0.293
Primary vs. Recurrent	0.785	0.301	0.516
Single vs. Multifocal	0.264	0.536	0.231
Size <3 cm vs. >3 cm	0.416	0.211	0.734
no CIS vs. CIS	0.209	0.475	0.640
No ReTUR vs. Re-TUR	0.854	0.586	0.489

*RFS, Recurrence Free Survival; PFS, Progression Free Survival; CSS, Cancer Specific Survival; Pts: patients. *Kaplan-Meier method and Log-rank testing. Data are expressed as p-values.*

that Re-TUR would have yielded an almost 45.8% rate of recurrent/residual disease also in Group B, the discrepancy in the rate of recurrent/residual disease between Re-TUR (45.8%) in group A and post-BCG bladder biopsies in group B (15%) would speak for the ability of BCG induction cycle to eradicate limited recurrent/residual disease in many cases. BCG treatment has been shown to modulate the urinary expression of multiple molecules regulating proliferative and angiogenic activity (13). Such cytotoxic, pro-apoptotic, and hypoxic effects of BCG would explain its ability to eradicate at least minimal residual tumors after the initial TURBT, thus explaining our two groups having similar rate of BC at bladder biopsies after the BCG induction cycle. Indeed, Oosterlinck et al. already demonstrated that more than 50% of exophytic Ta-T1 tumors (<10 mm) regressed after 6 weeks of BCG treatment (14), and Mack et al. (15) showed that BCG is able to ablate residual disease in a marker lesion study.

The most relevant question however remains whether Re-TUR improves RFS, PFS and CSS. The first RCT addressing this issue was carried out by Divrik et al. (16) who tested the role of Re-TUR in patients with T1 BC scheduled for adjuvant treatment with intravesical Mitomycin-C after initial TURBT. Re-TUR pointed out NMIBC in 33.3% of cases and upstage to T2 disease in 7.6% of cases.

Of note, Kaplan-Meier curves indicated a significant benefit in RFS and PFS but not in CSS for patients having undergone Re-TUR. Multivariable analysis stated that tumor number, tumor grade and Re-TUR were all significant independent predictors of disease recurrence, whereas tumor size and Re-TUR were significant independent predictors of disease progression. Though such findings would suggest Re-TUR to improve RFS and PFS, it is worth mentioning that this study included both low-grade and high-grade T1.

In a large retrospective study, Sfakianos et al. (17) compared RFS and PFS of patients they treated with intravesical BCG for high-risk NMIBC who had (n = 894) or had not (n = 127) undergone Re-TUR. Kaplan-Meier curves showed that Re-TUR provided a significant advantage in both RFS and PFS; multivariable analysis established that Re-TUR was the only significant predictor of recurrence at 5 years. Unfortunately, also this study suffers the biases of including tumors of different stages (Ta and T1) and grades (high-grade and low-grade), as well as not having evaluated at all the impact of prognostic factors on disease outcome.

The most interesting study defining the role of Re-TUR in a large yet homogeneous population of patients with high-grade T1 BC treated with intravesical BCG was carried out by Gontero et al. (10) Re-TUR, which was carried out in 935 (38%) of the 2451 patients, had a positive impact on

RFS, PFS, and CSS only if muscle was not present in the primary TURBT specimen. However, adjusting for the most important prognostic factors, Re-TUR in the absence of muscle had a borderline significant effect on RFS, PFS, and CSS, whereas Re-TUR in the presence of muscle in the primary TURBT specimen did not improve the outcome for any of these endpoints.

In agreement with Gontero's findings, we found that, in a homogeneous population of completely resected high-grade T1 BCs treated with BCG, Re-TUR did not improve RFS, PFS, and CSS.

Whether or not this finding is due to the efficacy of BCG treatment remains speculative.

Independently on such speculation, our data further support Gontero's conclusions that Re-TUR can be avoided in high-grade T1 BC providing it has been completely resected and the muscle is clearly visible and free of disease. The potential clinical relevance of such findings is obvious, as avoiding unnecessary Re-TURs means a significant reduction of both patient discomfort for the additional surgical procedure and healthcare costs.

The main limitations of our study was the absence of randomization, as the decision not to perform Re-TUR was a patient's informed choice. However, data were prospectively collected, and the two study groups had similar characteristics. Another study limitation was the relatively small sample size, but a single center study focusing on high-grade T1 tumors only should, however, guarantee for consistency in patients' population and study methodology.

CONCLUSIONS

Our study findings question the role of repeat TUR in case of a completely resected (muscle available and disease-free) high-grade T1 BC as this procedure might not translate into a better oncological outcome. In view of patient discomfort and healthcare costs associated with Re-TUR, the role of this procedure should be further investigated, ideally into the framework of a randomized study.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

BC, MC, FF, FS, EC-D, RA, GC, and LC participated in the study design. BC and MC performed the experiment. BC, MC, FF, FS, EC-D, RA, GC, and LC were involved in data collection and data interpretation. BC, MC, and FF participated in the statistical analyses. BC, RA, and LC wrote the manuscript. All authors read and approved the final manuscript.

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OSblca: A Web Server for Investigating Prognostic Biomarkers of Bladder Cancer Patients

Guosen Zhang[1†], Qiang Wang[1†], Mengsi Yang[1], Quan Yuan[1], Yifang Dang[1], Xiaoxiao Sun[1], Yang An[1], Huan Dong[1], Longxiang Xie[1], Wan Zhu[2], Yunlong Wang[3] and Xiangqian Guo[1]*

[1] Cell Signal Transduction Laboratory, Department of Preventive Medicine, Bioinformatics Center, School of Basic Medical Sciences, School of Software, Institute of Biomedical Informatics, Henan University, Kaifeng, China, [2] Department of Anesthesia, Stanford University, Stanford, CA, United States, [3] Henan Bioengineering Research Center, Zhengzhou, China

Bladder cancer (BC) is one of the most common malignant tumors in the urinary system. The discovery of prognostic biomarkers is still one of the major challenges to improve clinical treatment of BC patients. In order to assist biologists and clinicians in easily evaluating the prognostic potency of genes in BC patients, we developed a user-friendly Online consensus Survival tool for bladder cancer (OSblca), to analyze the prognostic value of genes. The OSblca includes gene expression profiles of 1,075 BC patients and their respective clinical follow-up information. The clinical follow-up data include overall survival (OS), disease specific survival (DSS), disease free interval (DFI), and progression free interval (PFI). To analyze the prognostic value of a gene, users only need to input the official gene symbol and then click the "Kaplan-Meier plot" button, and Kaplan-Meier curve with the hazard ratio, 95% confidence intervals and log-rank *P*-value are generated and graphically displayed on the website using default options. For advanced analysis, users could limit their analysis by confounding factors including data source, survival type, TNM stage, histological type, smoking history, gender, lymph invasion, and race, which are set up as optional parameters to meet the specific needs of different researchers. To test the performance of the web server, we have tested and validated its reliability using previously reported prognostic biomarkers, including *KPNA2*, *TP53,* and *MYC etc.*, which had their prognostic values validated as reported in OSblca. In conclusion, OSblca is a useful tool to evaluate and discover novel prognostic biomarkers in BC. The web server can be accessed at http://bioinfo.henu.edu.cn/BLCA/BLCAList.jsp.

Keywords: bladder cancer, prognostic biomarker analysis, web server, kaplan-meier curve, cox regression model

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University of California,
San Francisco, United States

***Correspondence:**
Xiangqian Guo
xqguo@henu.edu.cn

†These authors have contributed equally to this work

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INTRODUCTION

As one of the most common malignant tumors of the urinary system, bladder cancer (BC) is estimated to cause about 549,393 new cases and 199,922 deaths worldwide in 2018 (1). Based on the clinic-pathological features, BC could be classified into two types: non-muscle invasive tumor (NMIBC, 70–80% of BC patient) and muscle-invasive tumor (MIBC, 20–30% of BC patient) (2, 3). Due to the relatively high rate of local recurrence and metastasis in MIBC patients, the treatment outcome is still poor, and the survival rate is lower than that of NMIBC patients. Although NMIBC patients have better survival rates than MIBC, 30–50% of NMIBC patients experience cancer recurrence (4). One of the major challenges to improve clinical outcomes of BC patients is to screen novel biomarkers for diagnosis and prognosis (5).

In recent years, a large number of prognostic biomarkers including DNA markers and protein markers have been reported (6–8). Some of the prognostic biomarkers, especially the ones involved in biological processes, are useful to identify high-risk patients, and could be used to predict the prognosis and treatment response. However, few biomarkers have been translated into clinics due to the lack of independent validation (5, 9, 10). With the advance of high through-put technologies, more and more studies analyzed the gene expression of cancer samples and uploaded these data on public databases such as The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). These data offer opportunities for the biomarker discovery, validation, and clinical application (11, 12). Unfortunately, until now, this convenient online tool is still unavailable to clinicians and biologists to evaluate and verify the prognostic value of the genes of interests in different datasets for BC.

To solve this problem, we developed an online web server named OSblca, which consists of gene expression profiles and relative clinical information of 1,075 bladder cancer patients from seven independent cohorts collected from TCGA and GEO databases. This web server enables researchers and clinicians to analyze the prognostic value of a gene of interest and accelerates the development of prognostic biomarkers.

METHODS

Datasets Collection

Gene expression profiles and clinical follow-up information of bladder cancer patients were collected from TCGA and GEO databases. For TCGA dataset, level-3 gene expression profiling data (HiSeqV2) and clinical information of BC samples were downloaded in April 2018. In order to collect the relative datasets from GEO, keywords including "bladder cancer," "prognosis," "survival," and "gene expression" were used to search in GEO database. Next, manual checks of the availability of data of mRNA expression, clinical survival information and at least 50 patients were performed.

Development of OSblca

The OSblca web server was developed by Java script, and hosted by Tomcat 7.0 on Windows 2008. The database system that stores the gene expression and clinical data was handled by SQL Server 2008. The R package "RODBC" is used as a middleware to connect R and SQL. The input of OSblca web server must be the official gene symbol from NCBI (https://www.ncbi.nlm.nih.gov/). The outputs include Kaplan Meier (KM) survival curves, Hazard ratio (HR with 95% confidence interval) and log-rank *P*-value that are produced by R package "survival" (https://CRAN.R-project.org/package=survival). A gene could be regarded as a potential prognostic biomarker for BC patients when the log-rank *P*-value is < 0.05. OSblca can be accessed at http://bioinfo.henu.edu.cn/BLCA/BLCAList.jsp. A web server architecture diagram is presented in **Figure 1A**. The screenshot of the web server interface and the result are shown in **Figure 1B**.

Validation of Previously Published Prognostic Biomarkers in OSblca

In order to validate the performance of prognostic analysis in our web server, prognosis biomarkers for BC were searched in PubMed using the keywords "bladder cancer," "survival," "gene expression," "biomarker," and "prognosis." The prognostic capabilities of these genes were evaluated in all cohorts, and all cutoff values in "splitting the patients" were tested in each cohort to get the best cutoff value.

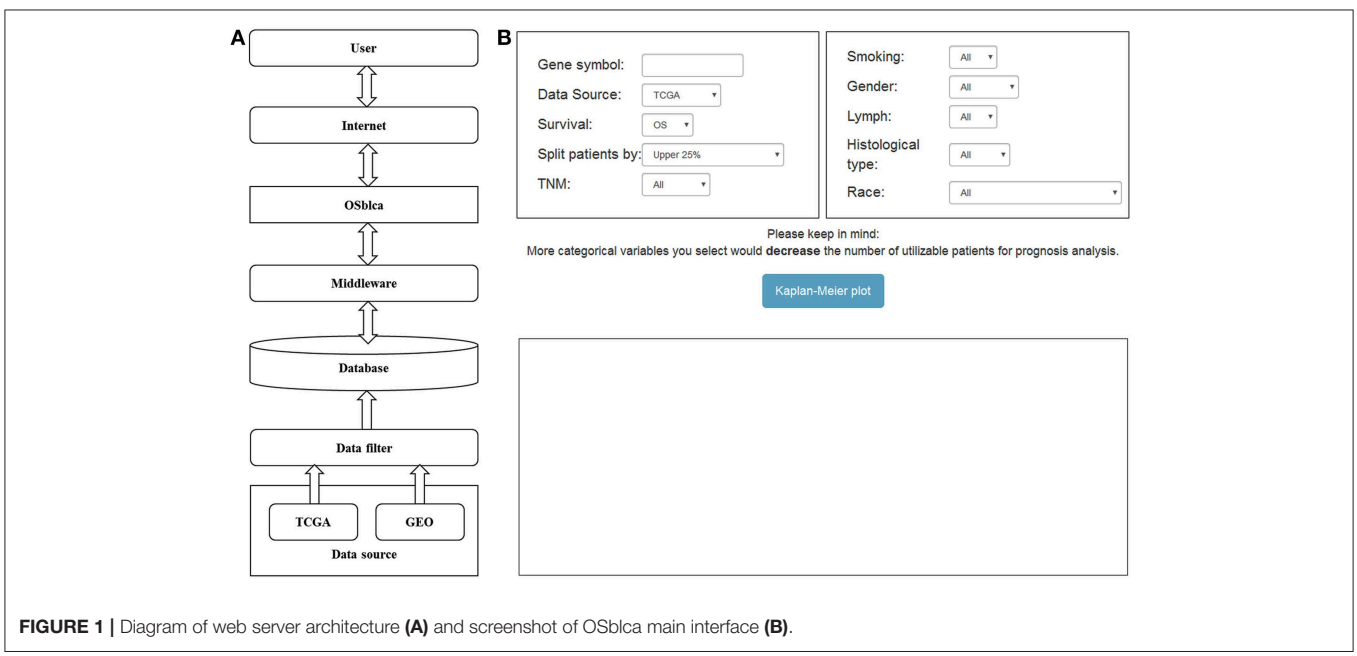

FIGURE 1 | Diagram of web server architecture **(A)** and screenshot of OSblca main interface **(B)**.

RESULTS

Clinical Characteristics of the Patients in OSblca

According to our criteria, in total 1,075 unique bladder cancer patients were collected from seven data sets including one TCGA cohort and six GEO cohorts. Survival information including overall survival (OS), disease specific survival (DSS), disease free interval (DFI), progression free interval (PFI) were gathered. No patient was lost to follow-up. Of the above, 935 patients have overall survival information, and the median overall survival time is 25.03 months. We also collected age, TNM stage,

TABLE 1 | Clinical characteristics of the BC patients collected in OSblca.

Data source	Platform	Sample size	Age	No. of death	Media (OS)	Gender (% male)	Stage (%I/II/III/IV/NA[a])	Never smokers (%)	Survival terms
TCGA	Illumina HiSeqV2	407	69 ± 11	155	16.93	73.71	0.49/31.70/34.40/32.92/0.49	26.78	OS, DSS, DFI, PFI*
GSE13507	GPL6102	165	65 ± 12	69	36.57	–	48.48/15.76/11.52/10.30/13.94	–	OS
GSE19915	GPL3883/ GPL5186	140		24	–	–	69.29/12.14/15.00/2.14/1.43	–	DSS
GSE31684	GPL570	93	69 ± 10	65	31.31	71.12	16.13/18.28/45.16/20.43/0.00	19.35	OS, DSS, DFI, PFI
GSE32548	GPL6947	130	70 ± 11	25	53.77	76.15	70.00/29.23/0.00/0.00/0.77	–	OS, DSS, DFI, PFI
GSE48075	GPL6947	73	69 ± 10	45	30.40	–	–	–	OS
GSE48276	GPL14951	67		31	34.10	80.60	2.99/8.96/25.37/53.73/8.95		OS, DSS
Total		1075	68 ± 11	414	25.03	74.46	29.64/23.26/23.85/20.86/3.39	11.81	

[a]*NA, Not Available; "–," no data; *DFI and PFI were defined by (13).*

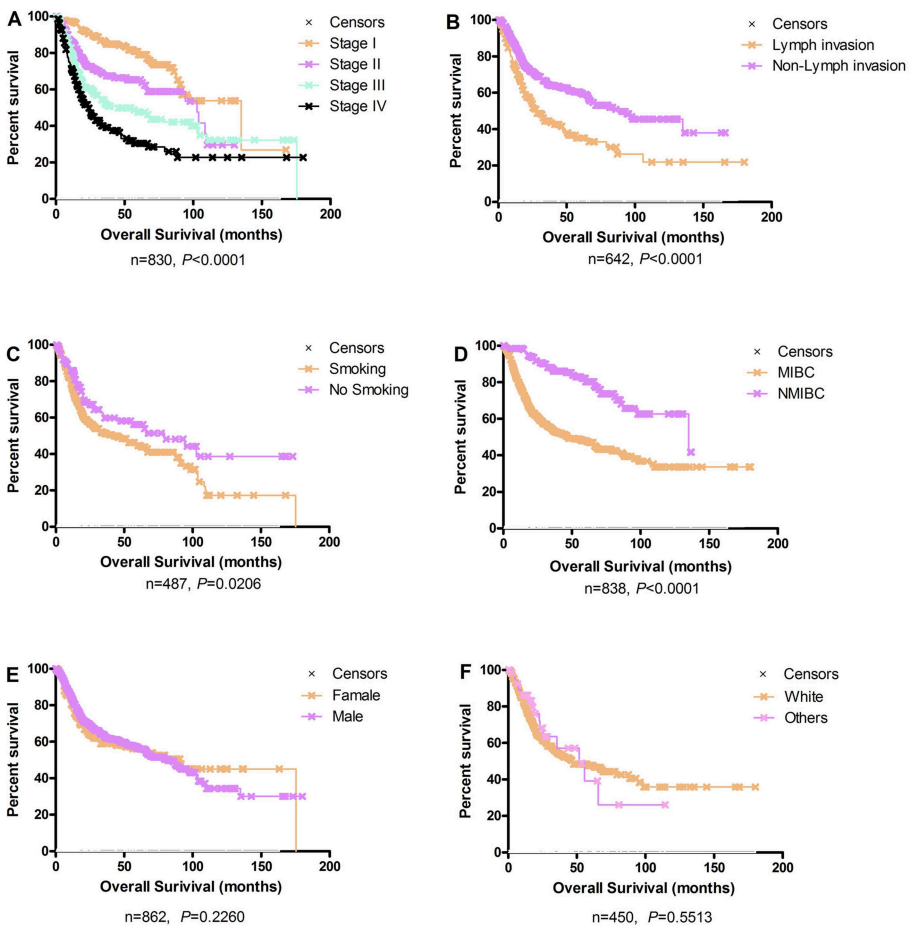

FIGURE 2 | Survival analysis of clinical characteristics of the bladder cancer patients in OSblca. **(A)** TNM stage; **(B)** Lymph invasion; **(C)** Smoking history; **(D)** Histological type; **(E)** Gender; **(F)** Race.

histological type, gender, smoking history, lymph invasion and race as confounding clinical factors. The average age is 68 ± 11. Distribution of TNM stages is as follows: stage I (n = 287, 29.64%), stage II (n = 233, 23.26%), stage III (n = 239, 23.85%), and stage IV (n = 209, 20.86%). The ratio of male to female of patients was close to 3:1. A summary of clinical properties for each dataset is presented in **Table 1**.

Survival Analysis of BC Patients Based on Clinical Characteristics

The Kaplan-Meier plots for the bladder cancer patients in OSblca stratified by TNM stage, histological type, gender, smoking history, lymph invasion, and race are presented in **Figure 2**. In these 1,075 patients, TNM stage, smoking history, lymph invasion, and histological type were significantly associated with overall survival ($P < 0.0001$, $P = 0.0206$, $P < 0.0001$, and $P < 0.0001$, respectively), which were consistent with previously reports (14–16). Nevertheless, gender and race showed no significant association with overall survival ($P = 0.2260$ and $P = 0.5513$).

Usage of OSblca

The main function that OSblca provides is to evaluate and verify the prognostic value for a given gene. "Gene symbol," "Data source," "Survival," and "Split patients" are set as the four main parameters. The input dialog box of "Gene symbol" is on the upper left of the OSblca page (**Figure 3A**). A red prompting message will show up when the input is not an official gene symbol. "Data source" provides eight options including independent analysis in one of seven cohorts and in a combined cohort consisting of all the BC patients from seven cohorts. The users can choose to evaluate the prognosis of a given gene in an individual cohort or in a combined cohort according to their needs. Under "Survival" option, four prognostic terms including OS, DSS, DFI, and PFI are provided. In the "Split patients" dialog box, user can select different thresholds of gene expression levels to divide patients into two subgroups for input gene. After then, by clicking the "Kaplan-Meier plot" button, OSblca server will take the request and return the analysis results, which are graphically displayed and presented with HR, 95% CI and log-rank P-value (**Figure 3B**).

In order to meet the specific needs, six confounding clinical factors including TNM stage, smoking history, gender, lymph, histological type, and race, were set as optional filter factors in the prognostic analysis. As showed in **Figure 3A**, each factor has 2–5 options for users to choose from.

Validation of Previously Published BC Biomarkers

To test the reliability of prognosis prediction in our web server, we evaluated 21 prognostic biomarkers from 16 previously reported literatures in the OSblca web server, including *KPNA2*, *TP53*, and *MYC* (17–32). As shown in **Table 2**, 17 out of 21 (82%) previous reported prognostic biomarkers were showed to have

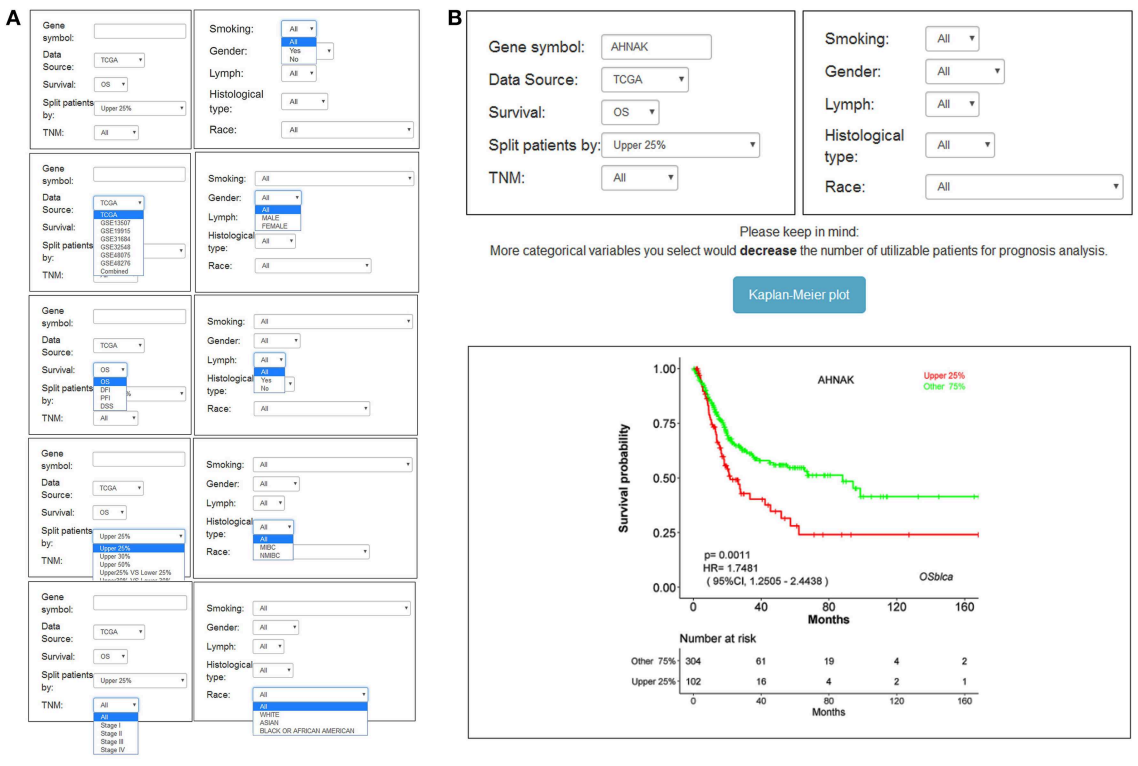

FIGURE 3 | Input and output interface of OSblca. **(A)** The options of main input parameters and clinical factors of OSblca; **(B)** The output interface of OSblca.

TABLE 2 | The validation of previous reported prognostic biomarkers in OSblca.

Gene symbol	Detection level	Case	Independent validation	In reference			In OSblca		
				P-value	HR	References	*P*-value	HR	Cut off
KPNA2	Protein	611	Yes	0.030	1.38	(17)	0.001[a]	1.42	Upper 25%
HYAL1	Protein	220	Yes	0.019	1.76	(18)	0.021[a]	1.29	Upper 25%
TP53	Protein	152	No	<0.001	–	(19)	0.037[a]	0.77	Upper 25%
MYC	Protein	132	No	0.020	–	(20)	0.050[a]	1.25	Upper 25%
RPS6	Protein	132	No	<0.010	–	(20)	0.007[a]	0.71	Upper 25%
JMJD2A	Protein	129	No	0.033	–	(21)	0.026[a]	0.63	Upper 25%
MKi67	Protein	115	No	<0.050	–	(22)	0.021[a]	1.29	Upper 25%
RRM1	Protein	84	No	0.001	–	(23)	0.000[a]	1.68	Lower 25%
MMP2	mRNA	41	No	<0.05	–	(25)	0.039[a]	1.26	Upper 25%
CDH2	mRNA	181	No	<0.001	–	(26)	0.038[a]	1.26	Upper 25%
PTGS2	Protein	273	No	0.027	0.65	(24)	0.050[b]	0.72	Upper 25%
CDH3	mRNA	181	No	<0.010	–	(26)	0.041[c]	2.30	Upper 25%
MDM2	Protein	84	No	<0.050		(27)	0.045[d]	1.89	Upper 25%
CCND3	Protein	157	No	<0.030	–	(28)	0.039[c]	2.32	Upper 25%
CCND2	Protein	57	No	0.042	–	(28)	0.047[f]	1.67	Lower 25%
LGALS3	mRNA	165	Yes	<0.001	–	(29)	0.016[e]	0.61	Lower 25%
USP28	Protein	206	Yes	<0.001	–	(30)	0.048[c]	0.29	Lower 30%
							0.014[d]	0.38	Lower 30%
DIABLO	Protein	84	No	<0.050	–	(31)	0.239[g]	0.87	NA
RB1	Protein	311	No	0.030	–	(25)	0.898[g]	1.02	NA
FGFR3	mRNA	114	No	0.035	–	(32)	0.462[g]	0.92	NA
CCND1	Protein	157	No	<0.020	–	(28)	0.997[g]	0.98	NA

[a] *Significant P-value validated in a combined cohort (OS);*
[b] *Significant P-value validated in a combined cohort (DSS);*
[c,d,e,f] *Significant P-value validated in dataset GSE32548, GSE48075, TCGA, and GSE13507, respectively;*
[g] *No significance P-value validated in any cohorts, "–" means no HR data, "NA" means not applicable.*

significant prognostic potency in OSblca, while the remaining four previously reported prognostic biomarkers did not reach significance in OSblca. Among the 17 validated prognostic biomarkers, 11 genes showed significant prognostic abilities in the combined cohort.

DISCUSSION

The discovery of prognostic biomarkers is a hot topic in translational research. In the current study, we present a convenient web server to assist researchers and clinicians to quickly screen and evaluate the prognostic value of genes in different cohorts of BC. As shown in a straightforward web interface, people without much bioinformatics experience can easily navigate OSblca to investigate genes of interests. In addition, users can perform survival analysis filtered by one or several factors according to the specific research purposes of their needs.

The validation of previously reported prognostic biomarkers in OSblac showed that our web tool is reliable and can be used in prognostic analysis for BC patients. Notably, 11 genes, such as *KPNA2* and *TP53*, were confirmed as prognostic biomarkers in the combined cohort, which indicated that these genes may be more widely applied as prognostic candidates for BC patients.

In summary, OSblca is a free online survival analysis web server that allows clinicians and researchers to rapidly analyze the prognostic value of a given gene in BC. We will keep updating OSblca to make it more powerful for the users.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: http://bioinfo.henu.edu.cn/BLCA/BLCAList.jsp.

AUTHOR CONTRIBUTIONS

GZ, QW, MY, and XG collected data, developed the server, and drafted the paper. QY, YD, XS, YA, and HD set up the server and performed the analyses. LX, WZ, and YW contributed to data analysis and paper writing. All authors edited and approved the final manuscript.

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Comprehensive Gene Expression Analysis in NMIBC Using RNA-seq Reveals New Therapy Strategies

Xiaoliang Chen[1†], Fuquan Jiang[1†], Chunshu Jia[2], Ming Liu[3], Yonghao Nan[4], Licheng Qu[2], Qingkuo Kong[2], Fangfang Hou[1], Wenshan Luo[1], Wanli Na[1], Xuefei Jin[1] and Jiufeng Tan[1*]*

[1] Department of Urology, China-Japan Union Hospital of Jilin University, Changchun, China, [2] Centre for Reproductive Medicine, Centre for Prenatal Diagnosis, First Hospital of Jilin University, Changchun, China, [3] Central People's Hospital of Siping City, Siping, China, [4] Department of Urology, the Frist Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Non-muscle invasive bladder cancer (NMIBC) patients often have fewer treatment options, and suffer the progression of disease due to mechanisms that are not clear, as well as due to its diversity. This study was designed to explore the molecular mechanism of bladder cancer through an RNA-seq. In addition to conventional analyses, we also simplified the network through modularization using the WGCNA algorithm, with the help of the topological overlapping matrix and hierarchical cluster tree, which are based on the PPI network of STRING. Furthermore, the hub genes were confirmed through survival analyses in the independent cohorts (n = 431). Among them, 15 genes were significantly associated with poor prognosis. Finally, we validated the results at mRNA and protein level using qRT-PCR, IHC and western blotting. Taken together, our research is important for the prediction, as well as the prospective clinical development of drug targets and biomarkers.

Keywords: NMIBC, modularization, WGCNA, PPI, biomarker

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***Correspondence:**
Xuefei Jin
25567726@qq.com
Jiufeng Tan
tanjf@jlu.edu.cn

[†]These authors have contributed equally to this work

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INTRODUCTION

Bladder cancer is a prevalent disease among the world, which is mainly attributed to smoking (1). Men are more likely to be affected than women, and morbidity increases with age (2). Bladder cancer grows through two distinct pathways: non-muscle invasive type and muscle invasive type. The majority of bladder patients are diagnosed through macroscopic hematuria, and diagnosis is confirmed after surgical resection, which is the primary stage of treatment (3). Although the 5 years survival rate as a result of current therapies is more than 80% for non-muscle invasive bladder cancer (NMIBC) patients, while a recurrence rate of nearly 70% results in patients being under lifelong surveillance and makes NMIBC the most expensive cancer from diagnosis to death (4, 5). Therefore, new therapeutic strategies are necessary to overcome these challenges.

Recent studies of bladder cancer based on gene expression profiles have gradually elucidated the molecular mechanism of the disease (6). Except for the transcriptional features have been discovered through earlier traditional microarrays, many molecular characteristics have been identified through integrative analyses (7, 8). Indeed, a lot of putative biomarkers and drug targets, including FGFR3, VEGF, CEBPA, and CCNE1, have been identified in different investigations (9–12). However, none of them have probed the regulation of their expression and of their associated genes, which could provide an extra perspective into the molecular mechanisms of disease progression.

In this study, we performed an RNA-seq on non-muscle bladder cancer patients who were subjected to surgical resection. After data processing, the reads were aligned with hg38 using STAR, and DESeq was used to filter the differentially expressed genes (DEGs). Then, we used these DEGs to execute functional annotation, including GO and KEGG enrichment analyses, which were further validated using GSEA. In order to simplify the network and identify functional clusters, modularization analysis was established through WGCNA and integrated with the PPI network from STRING, in order to model the dynamics of proteome changes. Afterwards, survival analysis was used to assess the clinical outcomes of hub genes, which are located as connections in each module. Finally, the results were confirmed through experiments, and it is hoped that they may be used as a reference for gene therapy for bladder cancer.

RESULTS

Identification of Differentially Expressed Genes (DEGs) and Functional Variation

DEGs were screened out using the DESeq package depending on read counts at transcription level, which were identified using an absolute $\log_2$FC value >1 and adjusted p-value of $<10^5$ as the statistical conditions for filtering. We obtained 885 DEGs, including 54 significantly upregulated genes and 831 significantly downregulated genes between the bladder cancer tissue and adjacent tissue. A volcano plot was used to visualize the results, in which hub genes and significantly changed genes were indicated (**Figure 1A**).

In order to further analyze the DEGs, we explored functional variation between the two groups using the clusterProfiler package. 430 GO terms were identified with an adjusted p-value of <0.01. The GOSemSim package was used to remove similar terms by keeping only one representative term, which resulted in 142 unique GO terms (13). The top 20 are shown in **Figure 1C**. Even though the extracellular matrix and organization structure were the most statistically changed functions, many terms involved in the immune response and activation, such as the regulation of lymphocyte activation, regulation of humoral immune response, adaptive immune response, and T cell activation, were also found. KEGG analysis also revealed many DEGs related to the downstream pathways of immune activation (**Figure 1B**), such as the PI3K-Akt signaling pathway, MAPK signaling pathway, and NF-kappa B signaling pathway. Most DEGs were downregulated in these processes. Recently, an unsupervised clustering by cytogenetic analysis divided NMIBC into two subtypes, no cytogenetic changes subtype (genomic subtype1, GS1) and another subtype with loss of 9q in chromosome (GS2). GS2 often appear in high grade tumors, and loss some regulators of AKT/PI3K/mTOR pathway. This may be why the dysfunction of AKT/PI3K pathway in NMIBC (14). In order to further verify the relationship between phenotype and functionally changed genes, we performed GSEA on the whole genome at transcription level. The transcripts of bladder cancer were remarkably associated with downregulated genes related to T and B cell receptor signaling pathways and their downstream pathways, which is in accordance with GO and KEGG enrichment analysis (**Figures 1D,E**).

Integrative Network Analysis Reveals New Functional Modules

An integrative analysis method was used to model the dynamics of proteome changes upon cancer progression, as described previously (15). In brief, we applied WGCNA to all DEGs in order to cluster the correlative proteins that had similar molecular functions or biological processes (16). Later, these proteins were superimposed onto the PPI network in order to identify functional modules. As a result, we identified 132 modules, with the number of proteins ranging from 17 to 2 (**Figure 2B**), and 117 out of the 132 modules were highly interconnected through their members (**Figure 2A**). Each module was annotated by known functional terms or signaling pathways. For instance, the modules were remarkably enriched in the immune reaction system including the T cell-mediated immune response (module 1, 13, 26, and 34), B cell-mediated immune response (module 11, 28, 34, and 38), mast cell activation (Module 6, 10, and 51) and natural killer cell mediated immunity (Module 39, 41, 47, and 75). Furthermore, some of the modules involved in cell invasion and migration processes also contributed to the progression of tumorigenesis, as commonly known, through mechanisms such as extracellular matrix organization (Module 4, 72) and the integrin-mediated signaling pathway (Module 32, 51). In summary, progression of bladder cancer is through the rebalanced regulation and extensive reprogramming of mutually connected functional modules.

Survival Analysis of Hub Genes

Based on the expression profile and clinical data of 431 bladder cancer samples from TCGA database, the clinical outcomes of hub genes that are indicated in **Figures 1**, **2** were evaluated through survival analysis. 15 out of the 62 hub genes were significantly associated with poor prognosis, and were either positively or negatively correlated with a higher risk and were either upregulated or downregulated with bladder cancer (**Figure 3**). Among the hub genes, CD3D was the core factor of the network, which was involved in the T cell receptor signaling pathway and was connected to T cell and mast cell activation. We computed the Pearson correlation of CD3D using 26,483 transcripts of 431 bladder cancer patients. CD2, CD6, and UBASH3A were the most positively correlated genes, while CD3D, and SCAMP1, MARVELD2, and KDM5B were the most negatively correlated. These genes might also be involved in the regulation of bladder cancer progression, and might also be candidate biomarkers or drug targets for the disease.

Initial Validation of Transcriptome Results Using qRT-PCR and IHC

In order to confirm the DEGs found through the experiment, total RNA of 24 paired tumor tissues were isolated for qRT-PCR validation. Twenty six target DEGs were selected as shown in **Figure 4**. Moreover, IHC was also performed to further validate the five target genes of patients who underwent surgical resection (**Figure 5**). In brief, the DEGs were successfully validated and

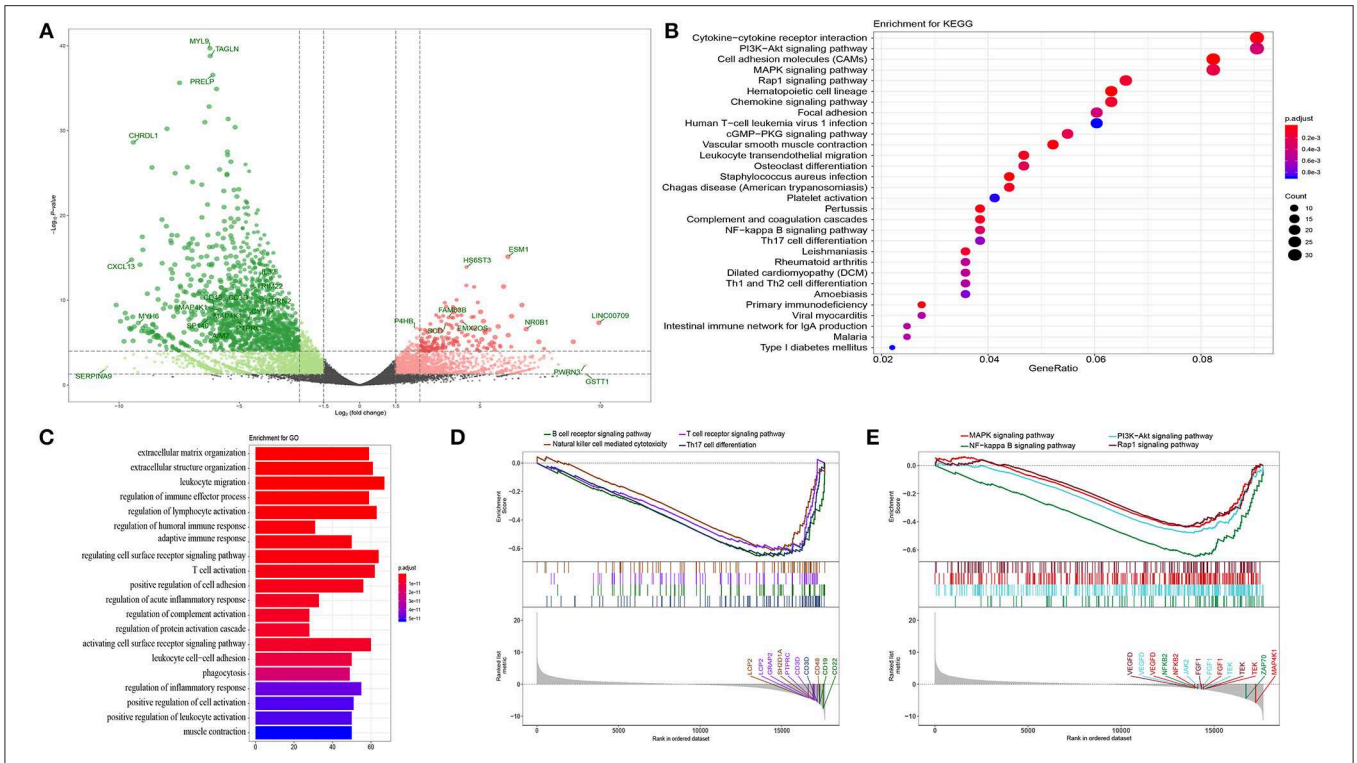

FIGURE 1 | Functional annotation. **(A)** Volcano plot of DEGs in bladder cancer compared with adjacent tissue. *P*-value were counted by the Wald test. Candidate hub genes were shown with different colors. **(B)** Bubble plot of top 30 enrichment of KEGG pathways. *P*-values were shown by different color, the size of bubble indicate the gene count of each pathway. **(C)** GO enrichments were arranged by top 20 significantly p values. **(D,E)** GSEA of whole transcription from RNA-seq, *p*-value was made by Kolmogorov-Smirnov test.

showed good correspondence with the analysis of transcriptome, indicating that the RNA-seq results were precise and reliable.

Signaling Pathway Validation Using Western Blotting

Finally, we wanted to confirm the signaling pathways at protein level. MAPKs are evolutionarily conserved kinases, ubiquitously expressed and regulate a wide range of biological processes, such as cell growth, differentiation and death (17, 18). In cancer, the MAPK signaling pathway can play a double role by either maintaining cell survival or impelling cell death, through different mechanisms (19). In this study, we found that Fibroblast growth factor receptor 1 (FGFR1), which is amplified in lung and breast cancer, was downregulated in bladder cancer samples compared with that of the controls (20, 21). FGFR1 genes are fused to TACC1 through interstitial deletions, which were also downregulated in our results ($\log_2 FC = -0.91$). The other three genes of the MAKP signaling pathway, PKCα, p21 Ras, and c-Fos, followed the same trend as that of FGFR1. More strikingly, protein phosphatase HePTP, which is a negative regulatory factor, also performed a similar action (**Figure 6**).

DISCUSSION

It is well-known that bladder cancer is the 11th most malignant tumor worldwide, and 70% of patients present with NMIBC. However, the exact biological functional variation during the progression of bladder cancer is still obscure. In order to provide deeper insights into the molecular mechanism involved in this process, we performed an RNA-seq on three paired bladder cancer patients who underwent surgical resection at China-Japan Union Hospital of Jilin University, and made a comprehensive analysis of the results, together with data from TCGA database. We identified core DEGs, significant biological processes, pathways, and validated our results using qRT-PCR, IHC and western blotting. In general, our work revealed an interlaced network presented by central modules that are involved in bladder cancer development, in which hub genes may play an indispensable role.

We sought for the expression patterns of transcripts and functional variations between bladder cancer tissue and adjacent tissues using RNA-seq, which produced a massive amount of data. In order to extract useful information from the large amount of data to explain the molecular mechanism of bladder cancer, in our study, we focused on two concepts. First, the DEGs were annotated by GO and KEGG pathway analyses, and the results involved functions related with immunity, cell adhesion and cancer. Furthermore, GSEA provided a good method of validating the functional annotations of the whole genome at transcription level rather than the DEGs. We also deciphered the complex network through modularization using WGCNA superimposed onto the PPI database of STRING. Each

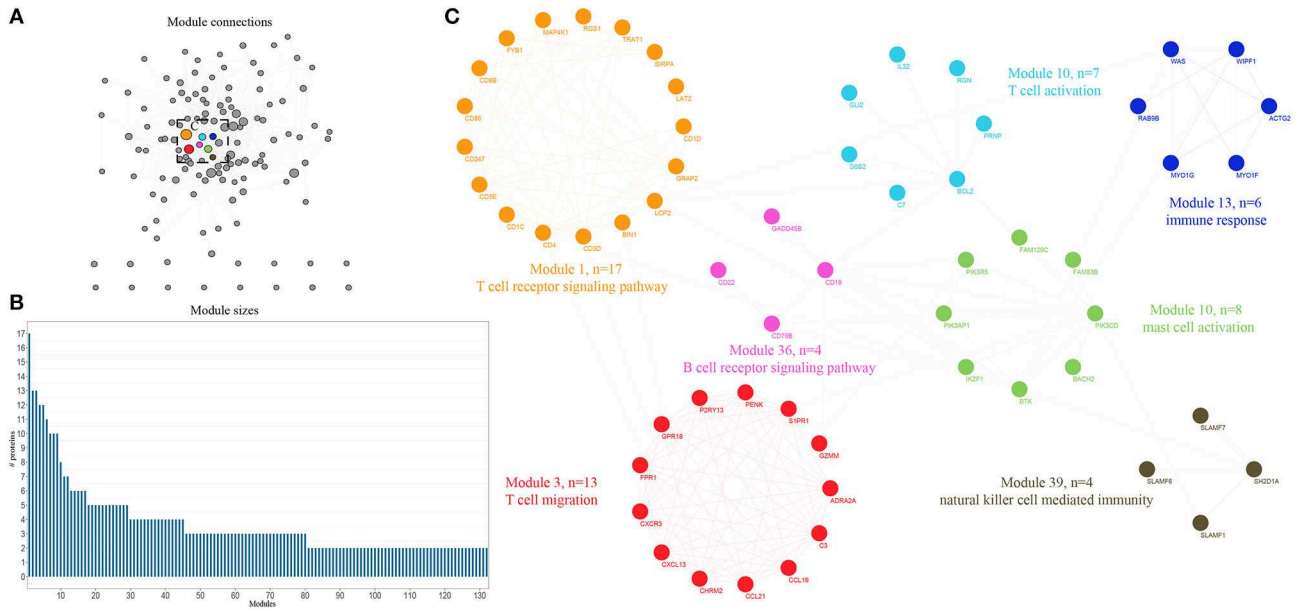

FIGURE 2 | Expression profiling of whole proteome reveals co-expression clusters and functional modules in bladder cancer. **(A)** Allocation of 132 modules. Each node represents the individual module and their interactions by the module size. Edges connect modules that share PPIs. Boxed modules are further enlarged in **(C)**. **(B)** Distribution of modules size, modules were identified by superimposition of proteins in bladder cancer onto the PPI network. The numbers of members from each module are exhibited in the figure. **(C)** Seven interconnected modules of immune reaction system derived from bladder cancer, with showing the protein names and representative functional terms.

module was facilitated through the hierarchical cluster tree and topological overlapping matrix, which echoed the annotated functions of GO and KEGG. Overall, the complicated network was simplified by modularization into modules, which made it easier for it to be learned by hub genes that were the connections among the modules. Second, the bladder cancer dataset obtained from TCGA was used to evaluate the clinical significance of the hub genes. Fifteen hub genes, including five upregulated and 10 downregulated, were associated with overall survival of patients, which indicates poor prognosis of bladder cancer. Among the hub genes, CD3D attracted our attention due to its location on the most important module. Pearson correlation was used to find the co-expression of CD3D and the expression pattern was assessed. Finally, partial hub genes were validated using qRT-PCR and IHC on specimens from the bladder cancer patients.

Along with the development and application of NGS technologies, a large number of sequencing data has been accumulated. However, we should be conscious of an analytical system that is so sophisticated that it is above our initial cognition. Fortunately, modern methodologies have provided us with a good way of simplifying complex networks, which include thousands of proteins that can be disassembled into several independent and correlated modules, and the hub genes of each module can be probed in detail. The active application of public databases promotes the elucidation of gene functions. As mentioned above, our study clearly presents the significant biological modules, pathways and hub genes involved in the progression of bladder cancer. However, some core genes might be shut out if they fail to be positioned in the modules, or have not been filtered out as DEGs, which may play an important role even though their expression does not greatly change during cancer progression. Therefore, we may have missed these genes in our analysis. Taken together, we systematically analyzed the molecular mechanism of functional variation in bladder cancer through biological modules and hub genes, which were confirmed using qRT-PCR, IHC, and western blotting. The revelation that they are involved in tumor progression could be used to design new strategies to treat aggressive carcinoma. For example, the downregulation of CD3D in bladder cancer samples and the T-cell receptors that are essential for the activation of T cell signaling, indicate a new therapeutic approach for bladder cancer. In addition, similarly, other hub genes may also prove to be useful drug targets and prognostic markers in gene therapies.

METHODS

Patients and Samples

All specimens were obtained from bladder cancer patients between April 2016 and December 2017 at China-Japan Union Hospital of Jilin University (Changchun, China), with the approval of the Ethics Committee. The samples were surgically resected followed by being treated with liquid nitrogen, and were then stored at −80°C. According to routine procedure, all samples were assessed using HE staining and diagnosis was made by three independent pathologists.

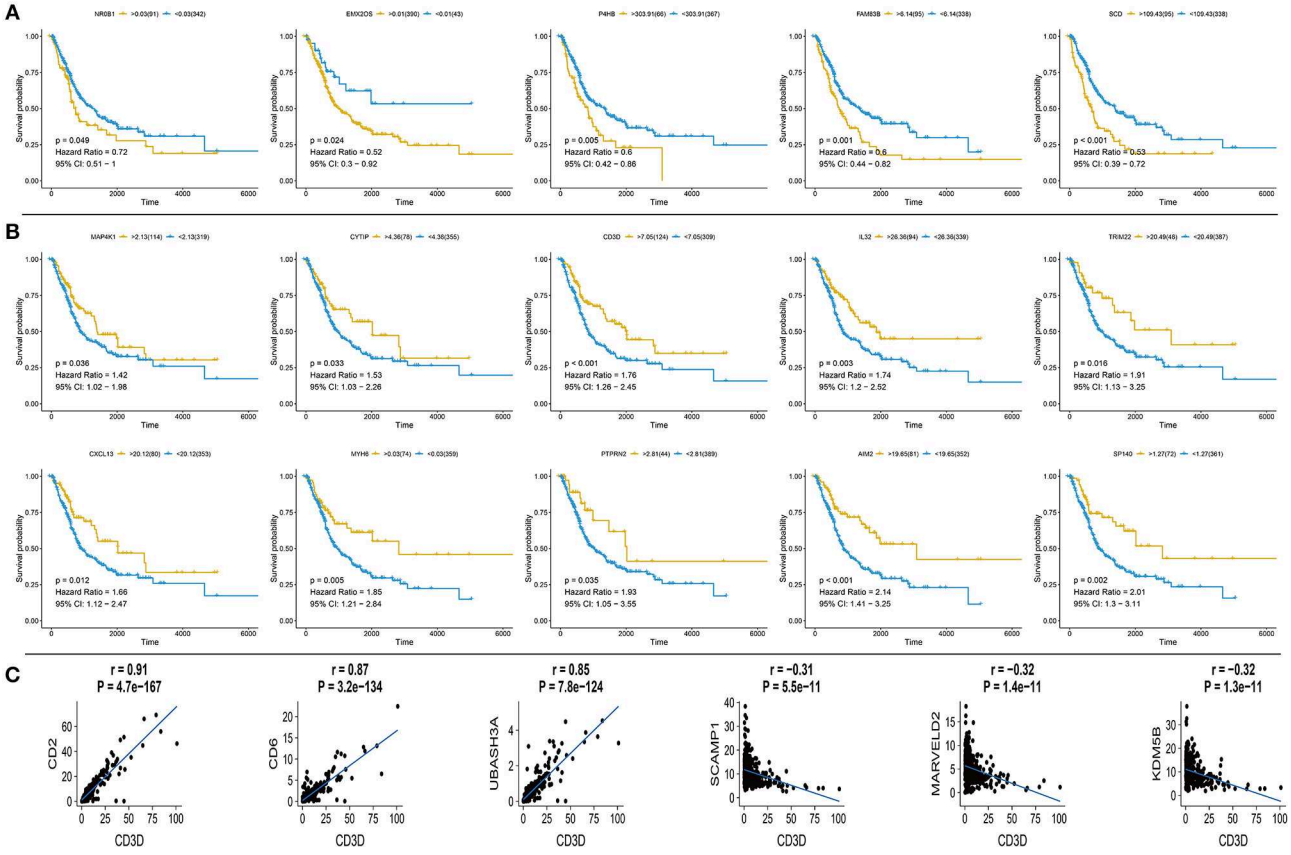

FIGURE 3 | Survival curve for hub genes of 433 TCGA bladder cancer samples and analysis of Pearson's correlation of CD3D **(A)** Five up-regulated hub genes with poor prognosis in bladder cancer, *p*-value, Hazard ration, and 95% CI are shown. **(B)** Ten down-regulated hub genes with poor prognosis in bladder cancer, p value, Hazard ration and 95% CI are shown. **(C)** Pearson's correlation of CD3D with top 3 positive and negative genes in bladder cancer from TCGA database, relevant gene expression versus change upon CD3D, *p*-value and correlation are shown.

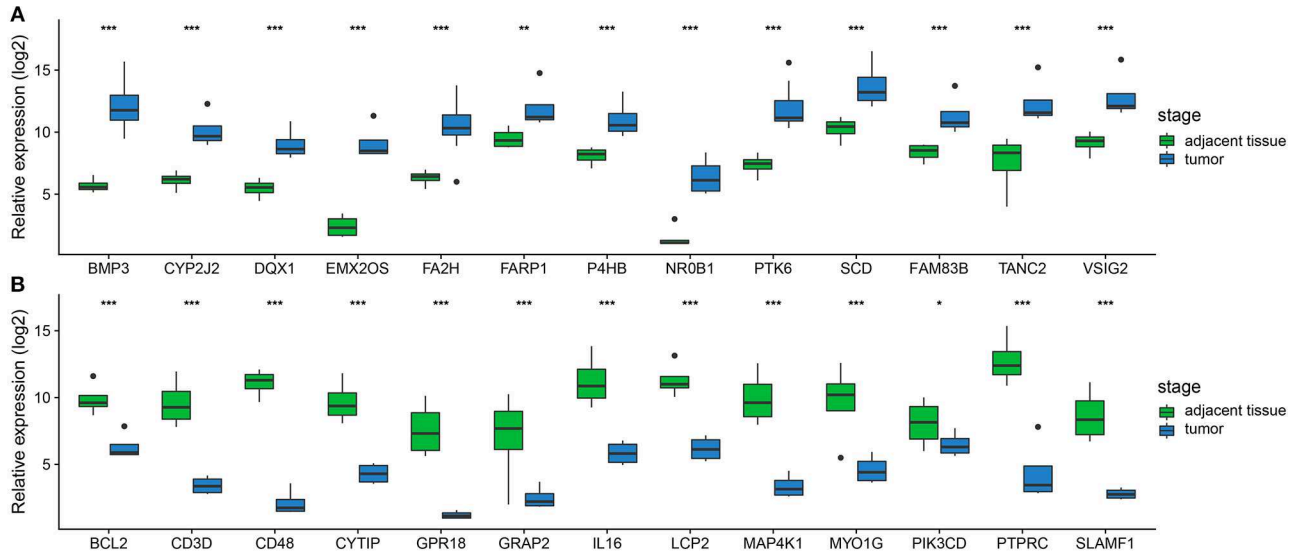

FIGURE 4 | Validation of DEGs by qRT-PCR. **(A,B)** Boxplots indicate the medians and dispersions of 24 bladder cancer and their adjacent tissue samples. *P*-values are counted by student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

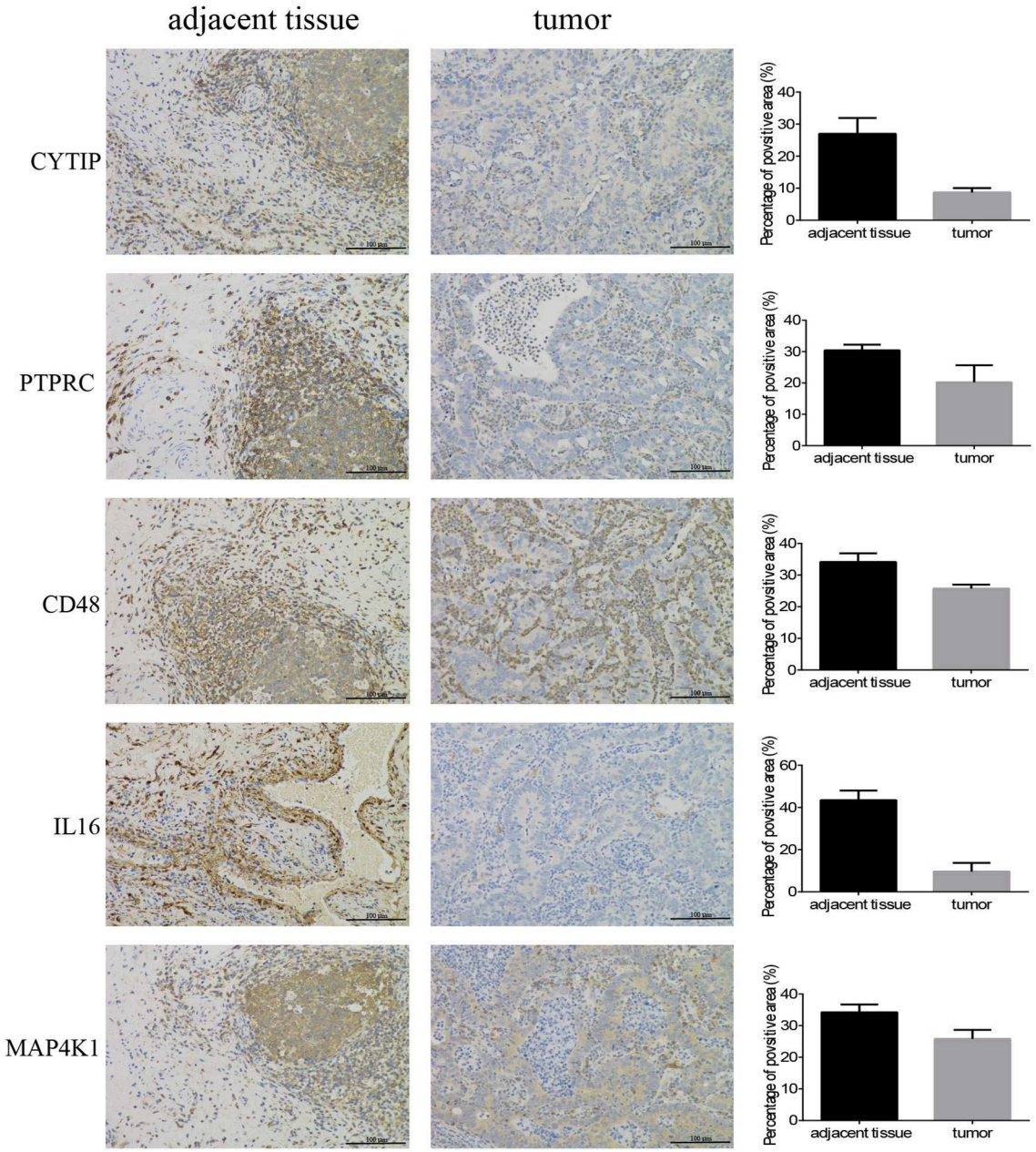

FIGURE 5 | Immunohistochemistry. Five hub genes expression in 12 pairs of bladder cancer and adjacent tissues (magnification 200×).

RNA-Seq and Data Processing

Total RNA of the three bladder cancer tissues and their paired adjacent tissues were isolated using Trizol reagent (Sangon Biotech, Shanghai, China), as described in other studies. The cDNA libraries were constructed using a custom protocol, which were sequenced using the Illumina Hiseq 2500 sequencer (Sangon Biotech, Shanghai, China). Raw data were uploaded to Sequence Read Archive (SRA) (PRJNA525544).

The adaptor sequences of raw reads were removed using cutadapt (22), then the clean reads were aligned to the human genome (hg38) using STAR (23). Prior to the next analysis, the R package, DESeq (24), was used to remove bad counts and filter the differential expression according to the conditions of an absolute $\log_2$FC value of >1 and an adjusted p-value of $<10^5$.

Functional Analysis

The DEGs were used to perform GO and KEGG analyses using the clusterProfiler package (25), and an adjusted p-values of <0.01 was considered as a significant event. Moreover, in order to deeply analyze functional variations between the bladder cancer tissue and their adjacent tissue, GSEA was utilized to discover the

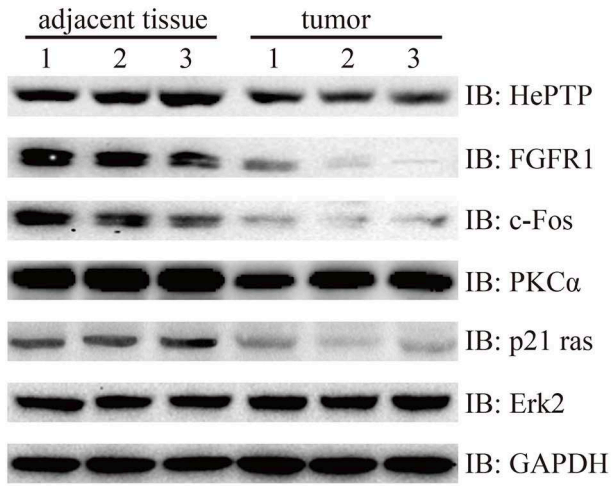

FIGURE 6 | Western blotting detection of MAPK signaling pathway. Lysates from three pairs of bladder cancer and adjacent tissues were subjected to western blotting with antibody to HePTP, FGFR1, c-Fos, PKCα, p21 ras, and Erk2. GAPDH is a reference gene.

molecular mechanism of the whole genome at transcription level, rather than the DEGs.

Network Analysis

All DEGs were used for the co-expression analysis using the WGCNA package (26) and were superimposed onto the PPI database of STRING (27). The co-expression analysis clusters were delineated using the dynamic tree cut package, with the minimum height for each module set to 0.2 (28). The overall trend of each module was based upon the eigengene, and the members of each module were collected through Pearson correlation from among DEGs and their interactors. Moreover, a topological overlapping matrix was also utilized to filter the PPI network (29). In the end, individual modules were annotated using clusterProfiler (25) and were visualized in Cytoscape (30).

Survival Analysis

The survival analysis was used to reveal the clinical outcomes of the hub genes in cancer prognosis. The expression profiles and clinical data of 431 bladder cancer patients were obtained from TCGA database using TCGAbiolinks (31). The 431 samples were split into a high expression group and a low expression group, according to the hub genes, using the survminer package for the best separation. A p-value of <0.05 was considered statistically significant and is shown in the results.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was used to verify the results of RNA-seq. The total RNA of 24 paired bladder tumors and their adjacent tissues were extracted using TRIzol. The genes of interest were then quantified through qRT-PCR using a One-Step qPCR Kit (Invitrogen, USA) and executed with a CFX ConnectTM Real-Time System (BIO-RAD, USA), according to the manufacturer's instructions. The results were analyzed through the $2^{-\Delta\Delta CT}$ method (32), with GAPDH as a reference gene.

Immunohistochemistry (IHC)

The specimens from the bladder patients who underwent surgical resection were cut to 4 μm thick sections, were then formalin-fixed and paraffin-embedded for IHC, as described previously (33). The primary antibodies used are as follows: CD48 (No.133506, Abcam), MAP4K1 (No.33910, Abcam), IL16 (No.184161, Abcam), CYTIP (No.154847, Abcam), and PTPRC (No.40763, Abcam). Image Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, United States) was employed to measure the positive area of hub genes for quantitative analysis.

Western Blotting

The tissue samples were stored at −80°C for 16 h and lysed with Tissue Extraction Reagent I (Invitrogen, USA) supplemented with protease and phosphatase inhibitors. The BCA assay kit (Thermo Scientific, USA) was then used to measure protein concentration. In brief, the lysate proteins were separated using SDS-PAGE, followed by being transferred into PVDF membranes (Invitrogen, USA), then subjected to the general process of western blotting, according to the instructions of the manufacturers of the antibodies, purchased from CST and Santa Cruz: PKCα (#59754, CST), FGFR1 (#9740, CST), c-Fos (#2250, CST), HePTP (sc-271245, Santa Cruz), p21 Ras (#3965, CST), Erk2 (#9108, CST) and GAPDH (#5174, CST).

Statistical Analysis

All experiments were performed in triplicate, at least. For the analysis between two groups, the student's t-test was leveraged for comparison between tumor tissue and its adjacent tissue. Data are presented as mean ± SDs, except when indicated otherwise. A p-value of <0.05 is considered to be statistically significant.

DATA AVAILABILITY

The datasets generated for this study can be found in Sequence Read Archive (SRA), PRJNA525544.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of CIOMS. The protocol was approved by the institutional review boards of the China-Japan Union Hospital of Jilin University. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

XJ and JT conceived and designed the study. XC, FJ, CJ, and ML collected analyzed the data, XC and FJ wrote the manuscript. YN, LQ, QK, FH, WL, and WN collected the samples and revised the manuscript. All authors read and approved the manuscript.

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External Replication of Urinary Bladder Cancer Prognostic Polymorphisms in the UK Biobank

Nadezda Lipunova[1,2,3], Anke Wesselius[2], Kar K. Cheng[4], Frederik J. van Schooten[5], Jean-Baptiste Cazier[1,3], Richard T. Bryan[1] and Maurice P. Zeegers[1,2]*

[1] Institute of Cancer and Genomic Sciences, University of Birmingham, Birmingham, United Kingdom, [2] Department of Complex Genetics, Maastricht University, Maastricht, Netherlands, [3] Centre for Computational Biology, University of Birmingham, Birmingham, United Kingdom, [4] Institute for Applied Health Research, University of Birmingham, Birmingham, United Kingdom, [5] Department of Pharmacology and Toxicology, Maastricht University, Maastricht, Netherlands

Introduction: Multiple studies have reported genetic associations with prognostic outcomes of urinary bladder cancer. However, the lack of replication of these associations prohibits establishing further evidence-based research directions. Moreover, there is a lack of independent bladder cancer patient samples that contain prognostic measures, making genetic replication analyses even more challenging.

Materials and Methods: We have identified 1,534 eligible patients and used data on Hospital Episode Statistics in the UK Biobank to model variables of otherwise non-collected events on bladder cancer recurrence and progression. Data on survival was extracted from the Death Registry. We have used SNPTEST software to replicate previously reported genetic associations with bladder cancer recurrence ($N = 69$), progression ($N = 23$), survival ($N = 53$), and age at the time of diagnosis ($N = 20$).

Results: Using our algorithm, we have identified 618 recurrence and 58 UBC progression events. In total, there were 209 deaths (106 UBC-specific). In replication analyses, eight SNPs have reached nominal statistical significance ($p < 0.05$). Rs2042329 (*CWC27*) for UBC recurrence; rs804256, rs4639, and rs804276 (in/close to *NEIL2*) for NMIBC recurrence; rs2293347 (*EGFR*) for UBC OS; rs3756712 (*PDCD6)* for NMIBC OS; rs2344673 (*RGS5)* for MIBC OS, and rs2297518 (*NOS2*) for UBC progression. However, none have remained significant after adjustments for multiple comparisons.

Discussion: External replication in genetic epidemiology is an essential step to identify credible findings. In our study, we identify potential genetic targets of higher interest for UBC prognosis. In addition, we propose an algorithm for identifying UBC recurrence and progression using routinely-collected data on patient interventions.

Keywords: bladder cancer, SNP, replication, UK Biobank, prognosis

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****Correspondence:***
Nadezda Lipunova
nxl689@student.bham.ac.uk;
n.lipunova@maastrichtuniveristy.nl

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INTRODUCTION

Urinary bladder cancer (UBC) is a disease of great burden; yet the diagnosis, clinical management, and patient survivorship has changed little over the last few decades (1, 2). Genetic studies may provide important clues on biological pathways underlying the development of UBC. Importantly, advances in understanding what drives a favorable UBC prognosis

could aid in predicting patient outcomes. As a result, and informed and timely patient stratification would allow an individually-tailored cancer management plan, which is likely to better reflect patient needs than current group-level recommendations (3).

Multiple genetic associations with UBC prognostic outcomes (e.g., survival, recurrence) have been reported in the literature (Lipunova et al., under review). However, the number of potential genetic clues far exceeds the available resources for clinical and functional investigation. As such, the scientific community must take an approach of targeting most-promising associations first.

There are multiple ways to define clinical relevance of a genetic variant, including external replication to reduce the chance of false-positives (4, 5). However, replication of genetic associations includes many hurdles, such as a lack of independent participant cohorts with adequate sample sizes. Moreover, focus on a sub-phenotype (e.g., recurrence) makes it even more difficult due to required additional sources of data (e.g., hospital records).

Increased availability of population-based electronic health records can help to alleviate the burden of investigating diseases for which adequate sample sizes are difficult to acquire. UK Biobank is the largest population-based cohort in the United Kingdom and serves as a powerful resource for investigating genetic associations (6) and has not yet been widely used for investigating UBC. The presence of Hospital Episode Statistics (HES) in the UK Biobank offers an unprecedented opportunity to use these data to identify UBC recurrence and progression events, that are not a part of the usually-collected information.

In the current study, we have aimed to identify UBC patients in the UK Biobank and use HES statistics to construct prognostic events. We have further used this data to externally replicate previously reported genetic associations on UBC survival, recurrence, and age at the time of diagnosis.

MATERIALS AND METHODS

SNP Selection

We have aimed to replicate all SNPs that have been previously associated with UBC recurrence, progression, death (overall or cancer-specific), and age at the time of diagnosis. The polymorphisms were extracted from a recent review on prognostic UBC outcomes (Lipunova et al., under review). To capture any associations reported since the review, we have updated the list of SNPs by querying PubMed database for new articles using identical search terms to those used in the review (**Figure 1**). The search was limited to articles published in English language between 13th November 2018 and 19th February 2019. Eleven papers were identified in total, with one study being eligible for inclusion (7). Additionally, we have included associations for age at the time of diagnosis from a genome-wide association study (GWAS) previously carried out in the Bladder Cancer Prognosis Programme (BCPP) (8).

After removing duplicate entries, there were 69 SNPs to test for recurrence, 53 for survival, 20 for age, and 23 for progression (**Supplementary Tables 1–4**).

Study Population

UK Biobank is a population-based cohort in the UK, having collected genetic and clinical data on over 500,000 participants, aged 40–69 at the time of recruitment in 2006–2010. The design, data collection and processing are described in detail elsewhere (6, 9).

Our analysis was restricted to UBC patients (corresponding International Classification of Diseases (ICD) codes of C67.0, C67.1, C67.2, C67.3, C67.4, C67.5, C67.6, C67.7, C67.8, C67.9, D09.0 (ICD10) and 1880, 1882, 1884, 1886, 1888, 1889, 2337 (ICD9). To prevent bias from analyzing heterogeneous molecular UBC subtypes, histology was limited to the following ICD-O (ICD Oncology) codes: 8000 (Neoplasm), 8001 (Tumor cells), 8010 (Carcinoma), 8020 (Carcinoma, undifferentiated), 8050 (Papillary carcinoma), 8120 (Transitional cell carcinoma), and 8130 (Papillary transitional cell carcinoma).

HES contains admitted in-patient data starting with 1997 (10) and includes data on patients both under National Health Service (NHS) and private care. HES data is provided to the UK Biobank on an annual basis, covering the past financial year (starting 1st April of each year). In our analyses, the follow-up covers all in-hospital interventions registered until March 31st, 2017. Operative procedures use OPCS4 (Office of Population, Censuses and Surveys: Classification of Interventions and Procedures, Version 4) coding system.

In total, there were 1,534 UBC patients with clinical and genetic data available for analysis.

Outcomes

Age

Age at the time of diagnosis was modeled both as a continuous and categorical variable.

To replicate previous associations as accurately as possible, we have dichotomised age variables using the cut-off points reported in the original research articles ($\geq$/$<$50, 55, 60, 65, and 70 years, **Supplementary Table 3**).

Death

Death was modeled as an overall (death vs. no death) or a UBC-specific event (death vs. no death, when primary cause of death was assigned C67-(ICD10) or 188-related (ICD9) codes).

Recurrence

The events of bladder recurrence and progression are not part of the routinely collected data in the Cancer Registry, or other national/regional datasets. However, the HES in the UK Biobank make it possible to identify a fraction of these events using proxy data.

For recurrence, we have considered three conditions to be representative of an event (**Figure 2**). First, a transurethral resection of a bladder tumor (TURBT) (OPCS4 code M42) is regarded to be enough to signify a UBC event. Secondly, a time gap of longer than 4 months between chemotherapeutic treatments into urinary bladder (OPCS4 codes M494/M495) was considered to be substantial to correspond to two different events. Thirdly, we have assumed a UBC diagnosis if an examination of the urinary bladder (OPCS4 code M45) was

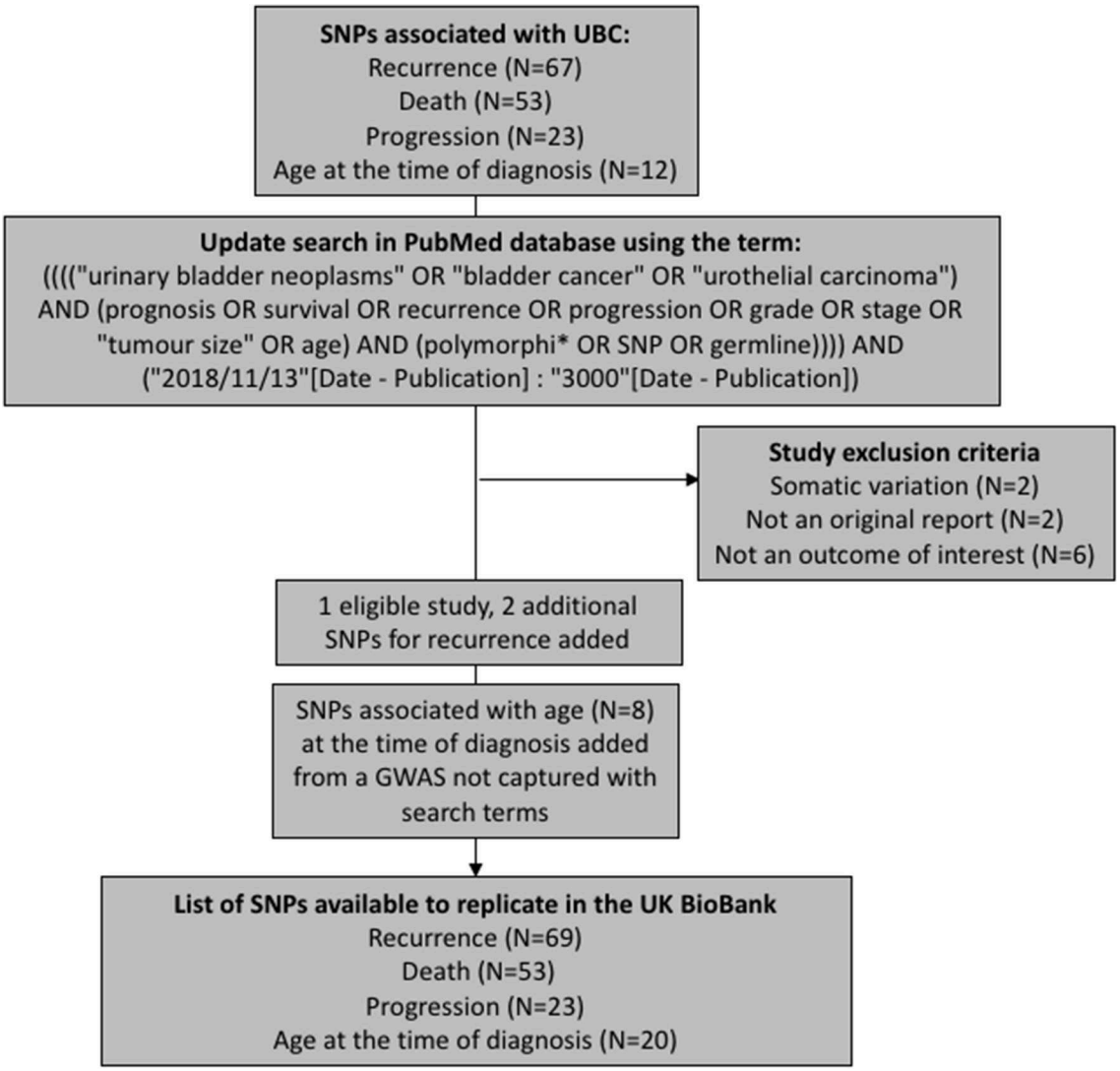

FIGURE 1 | Selection process of the SNPs used in replication analyses.

led by an intervention within 6 months. Relevant interventions were chemotherapeutic treatments into urinary bladder, cystectomy, radiotherapy, and chemotherapy (corresponding to OPCS4 codes of M494/M495, M34, X65, X72, X292, X298, X308, X352, respectively). Currently presented list of chemotherapy-related OPCS4 is not exhaustive, but rather based on interventions observed in our data. Further development of the algorithm is likely to adjust the list as needed.

Progression

In our framework, all events of progression are recurrences by default. However, we have considered adding additional criteria would allow distinguishing which recurrences were also representative of UBC progression. We have considered an event of UBC progression to have taken place if either a TURBT (OPCS4 code M42) or examination of the urinary bladder (OPCS4 code M45) was followed by interventions of cystectomy (OPCS4 code M34) and/or radiotherapy (OPCS4 code X65) within 6 months (**Figure 2**).

To prevent registration duplicates, two recurrence and/or progression events were considered independent of one another if time in between the records was >3 months.

Invasiveness at the Time of Diagnosis

Finally, UBC clinical management is heavily dependent on its' invasiveness at the initial diagnosis. A UBC diagnosis that was followed by either cystectomy or radiotherapy was considered to represent a muscle-invasive bladder cancer (MIBC), while the remaining diagnoses are held to be non-muscle-invasive bladder cancer (NMIBC) cases (**Figure 2**).

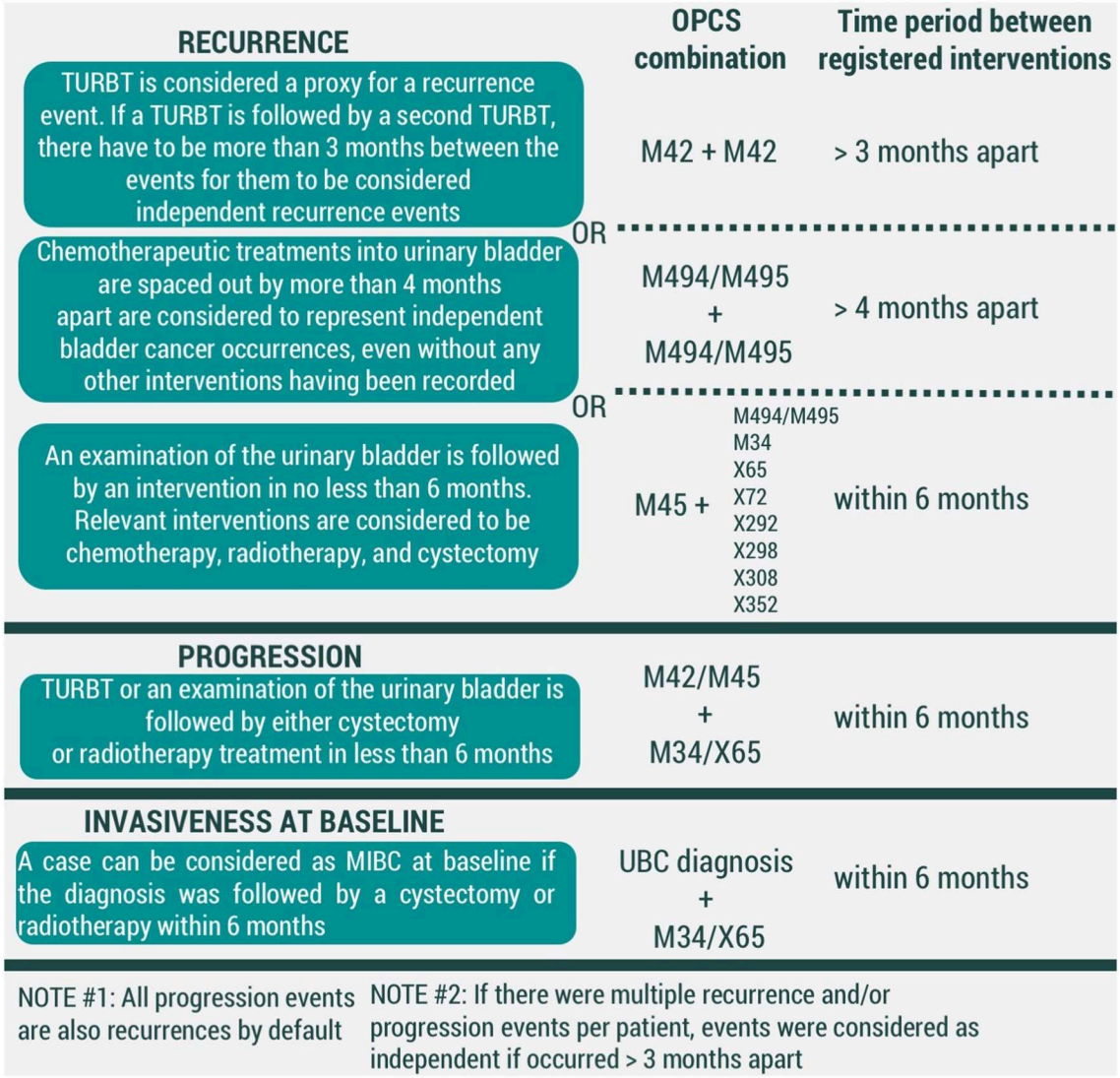

FIGURE 2 | Conditions for modeled events of UBC recurrence, progression, and invasiveness at baseline (MIBC-muscle-invasive bladder cancer, TURBT-transurethral resection of bladder tumor). All codes correspond to OPCS4 classification.

Ethics and Consent

All UK Biobank participants have provided informed consent. Current research has been conducted using the UK Biobank Resource under Application Number 42772.

Genotype Data Quality Control (QC) and Imputation

Detailed procedures on QC and imputation in the UK Biobank are described elsewhere (9).

To verify the high quality of all tested SNPs, we have extracted imputation accuracy measures (INFO scores) and MAF (minor allele frequencies) (**Supplementary Table 5**). INFO scores are computed to estimate the level certainty of imputed SNPs. The value ranges from 0 to 1, with estimates close to 1 representing SNPs imputed with high accuracy (11).

To avoid population stratification bias, we have restricted our sample to a homogenous group of White British participants, as previously identified by the UK Biobank team (9).

Statistical Analysis

To test for an association between selected SNPs and UBC recurrence, progression, death, and age, we have utilized SNPTEST (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html). To estimate Linkage disequilibrium (LD), an online tool was used (https://ldlink.nci.nih.gov/). LD defines the correlation between alleles in a given population. Due to some SNPs being in high LD, it might be difficult to establish which allele is representing the cause, as they are often inherited together. At the same time, linkage equilibrium suggests alleles are inherited independent of one another. Logistic regression

using allele dosages was applied to estimate odds ratios (OR) and corresponding confidence intervals (CI) for death, recurrence, progression, and categorical age events; while linear regression was used to estimate the effect of age as a continuous variable. All associations were tested under additive model of inheritance and adjusted for participant sex. To reduce multiple testing, analyses were ran for the outcome that resembled the originally-reported association most closely (e.g., if a variant has been associated with NMIBC recurrence, we have only tested NMIBC patients instead of the whole UBC sample). To better estimate the strength of evidence for replication results, we additionally included calculation of the Bayes Factor (BF). In simple terms, BF can be considered as a ratio of probabilities for two competing hypotheses (for example, the probability of a SNP being associated with an outcome vs. the SNP not influencing the outcome). The ratio provides an estimate that shows the extent of one hypothesis being more (or less) likely than the alternative one. In contrast, the generically-used frequentist approach (resulting in a *p*-value) evaluates the probability of data under a specific hypothesis, which alone does not provide indication of the association strength.

Variants in the replication were considered promising if the nominal statistical significance (*p*-value) has reached <0.05. Bonferroni adjustment per each outcome for multiple comparisons resulted in statistical significance level (*p*-value) to be 0.0007 for recurrence ($\alpha = 0.05/69$), 0.002 for progression ($\alpha = 0.05/23$), 0.0009 for survival ($\alpha = 0.05/53$), and 0.0025 for age ($\alpha = 0.05/20$).

TABLE 1 | Descriptive characteristics of the UBC patients from the UK Biobank.

	N	*p*-Value*
Sex	**1,534**	**<0.001**
Males (%)	1,197 (78.0)	
Females (%)	337 (22.0)	
Age [Mean (SD)]	61.3 (9.0)	
Death	**1,534**	**<0.001**
No (%)	1,325 (86.4)	
Yes (%)	209 (13.6)	
UBC-specific death	**1,534**	**<0.001**
No (%)	1,428 (93.1)	
Yes (%)	106 (6.9)	
Recurrence	**1,534**	**<0.001**
No (%)	916 (59.7)	
Yes (%)	618 (40.3)	
Progression	**1,534**	**<0.001**
No (%)	1,476 (96.2)	
Yes (%)	58 (3.8)	
NMIBC at baseline	**1,534**	**<0.001**
No (%)	114 (7.4)	
Yes (%)	1,420 (92.6)	

**Chi-square test for group independence.*
NMIBC, non-muscle-invasive bladder cancer; SD, standard deviation; UBC, urinary bladder cancer.

RESULTS

In total, 1,534 UBC patients were available for replication analyses of prognostic events (**Table 1**). Mean age of UBC patients was 61 years, and most were males (78%). Using our algorithm on HES data, we could identify UBC invasiveness at baseline, recurrent, and progressive events for UBC patients in the UK Biobank cohort. Majority of UBC cases were NMIBC (93%). Death was recorded for 209 (13.6%) patients, out of which 106 were UBC-specific. In addition, we estimate 618 patients (40%) have experienced a recurrence, and 58 (3.8%) have had a UBC progression.

In the replication analyses, eight SNPs have reached a *p*-value of <0.05 (**Table 2**). However, none of the variants remained significant after applying Bonferroni-corrections for multiple comparisons (corrected for each tested outcome).

Recurrence

Four of these SNPs were associated with bladder cancer recurrence. Rs2042329 (*CWC27*) was linked to an increased risk of UBC recurrence (OR = 1.26, 95% CI: 1.10; 1.48); while rs804256, rs4639, and rs804276, all located in/close to *NEIL2* were associated with NMIBC-only recurrence (OR = 1.23, 95% CI: 1.05–1.43; OR = 1.20, 95%CI: 1.03–1.39; OR = 1.17, 95% CI: 1.01–1.36, respectively). All SNPs that were associated with recurrence showed consistent direction, but were more modest in comparison to the original studies [HR = 1.54 (1.10–2.16) for rs2042329 (12), HR = 4.58 (2.61–8.02) for rs804256 (13), HR = 2.60 (1.68–4.03) for rs4639 (13), and HR = 2.71 (1.75–4.20) for rs804276 (13)].

Although SNPs rs804256, rs4639, and rs804276 all map to the same locus, LD values imply they are independent results (R^2 for rs804276 and rs804256 = 0.09; R^2 for rs804276 and rs4639 = 0.43; R^2 for rs4639 and rs804256 = 0.38).

Death

Three SNPs [rs2344673 (*RGS5*), rs3756712 (*PDCD6*), and rs2293347 (*EGFR*)] were associated with events of bladder cancer death, albeit in different subgroups. Rs2293347 (*EGFR*) was associated with lower death rates among all UBC patients (OR = 0.69, 95% CI: 0.47–0.99), rs3756712 (*PDCD6)* was significant for NMIBC patients (OR = 1.29, 95% CI: 1.02–1.63), and rs2344673 (*RGS5*) showed reduced rate of death among MIBC cases (OR = 0.22, 95% CI: 0.05–0.98).

In comparison to the original study, replicated SNPs in *PDCD6* showed effect in the same direction, but had a reduced estimate [HR = 5.11 (1.43–18.22) (14)].

However, inconsistency in direction of the effect was observed for SNPs in *EGFR* and *RGS5* [HR = 1.5 (1.0–2.3) for rs2293347 (15) and HR = 1.55 (1.15–2.11) for rs2344673 (16)].

Progression

Carrying a minor allele of rs2297518 in *NOS2* corresponded to a lower chance of UBC progression (OR = 0.56, 95% CI: 0.32–0.99). In the original study, rs2297518 was also associated with a lower risk of progression [HR = 0.21 (0.05–0.87) (17)].

TABLE 2 | Replication results that have reached p < 0.05 in the UK Biobank cohort.

Outcome	rsID	Locus	REF	EFF	info value	MAF, % (all)	*N* (total) (AA/AB/BB)	MAF, % (cases)	*N* (cases) (AA/AB/BB)	MAF, % (controls)	*N* (controls) (AA/AB/BB)	OR	(95% CI)	*P*-value	log10(BF)	Annotated gene
UBC recurrence	rs2042329	5q12.3	T	G	1	41	1,534 (255/739/540)	37	618 (88/284/246)	43	916 (167/455/294)	1.26	(1.10; 1.48)	0.001	1.56	CWC27
NMIBC recurrence	rs804256	8p23.1	T	C	0.99	34	1,420 (634.7/604.96/180.4)	37	607 (254.1/261.2/91.7)	32	813 (380.6/343.8/88.6)	1.23	(1.05; 1.43)	0.012	0.74	NEIL2
MIBC overall survival	rs2344673	1q23.3	G	A	1	12	123 (94/29/0)	4	26 (24/2/0)	14	97 (70/27/0)	0.22	(0.05; 0.98)	0.019	0.10	RGS5
NMIBC overall survival	rs3756712	5p15.33	A	C	0.99	38	1,420 (200.4/667.6/552.0)	33	184 (16.9/85.9/81.2)	38	1,236 (183.5/581.7/470.8)	1.29	(1.02; 1.63)	0.03	0.49	PDCD6
NMIBC recurrence	rs4639	8p23.1	A	G	0.99	43	1,420 (472.7/672.9/274.4)	46	607 (182.2/297.0/127.8)	41	813 (290.5/375.8/146.6)	1.20	(1.03; 1.39)	0.02	0.56	NEIL2
NMIBC recurrence	rs804276	8p23.1	G	A	0.99	41	1,420 (496.9/670.1/253.0)	44	607 (200.0/284.5/122.5)	40	813 (296.9/385.6/130.5)	1.17	(1.01; 1.36)	0.04	0.34	–
UBC overall survival	rs2293347	7p11.2	C	T	0.99	11	1,534 (1215.6/303.5/14.9)	8	209 (176.4/31.6/1.0)	11	1,325 (1,039.2/271.9/13.9)	0.69	(0.47; 0.99)	0.04	0.35	EGFR
UBC progression	rs2297518	17q11.2	G	A	1	19	1,534 (999/475/60)	12	58 (45/12/1)	20	1,476 (954/463/59)	0.56	(0.32; 0.99)	0.03	0.26	NOS2

BP, base pair; BF, Bayes' Factor; CI, confidence interval; EFF, effect allele; MAF, minor allele frequency; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; OR, odds ratio; REF, reference allele; rsID, polymorphism ID; UBC, urinary bladder cancer.

Bayes factor was highest for the variant associated with NMIBC recurrence in *CWC27*, reaching log10(BF) = 1.56. For all remaining SNPs, Bayes statistic indicates replication sample was low-powered (18), with log10(BF) ranging between 0 and 1.

DISCUSSION

In the current study, we describe an external replication of previously reported genetic associations for UBC recurrence, progression, death, and age at the time of diagnosis using HES data available the UK Biobank.

The aim of our study is 2-fold. Firstly, mining routinely-collected data for identifying complex phenotypes is inevitable to become a common practice. In the light of current needs, we propose an algorithm that identifies UBC recurrences and progression events via recorded interventions in a hospital setting. Current approach uses OPCS4 classification system, but we are confident applied assumptions can be translated to other globally-used systems (e.g., International Classification of Health Interventions, ICHI). We acknowledge identified prognostic events make up only a fraction of the true event volume, and are likely to be an underestimate. The extent of the underestimation requires testing the algorithm in an external cohort and is a necessary subsequent step in refining the currently-described approach. The level of underestimation is likely to vary for differed outcomes, as some events are arguably easier to identify (e.g., recurrence), while progression requires more detailed data and is subject to a higher level of underrepresentation. However, we saw an overestimation resulting in a greater rate of error and data misrepresentation. Moreover, inclusion of other clinically-relevant characteristics (tumor stage, grade) would increase the accuracy of modeled prognostic events. The provisioned release of such data in the UK Biobank (https://biobank.ctsu.ox.ac.uk/crystal/exinfo.cgi?src=future_timelines) will provide further opportunities of updating the algorithm. Naturally, our proposed approach and assumptions are subjective by nature and we encourage the expert field to contribute ideas to make the assumptions more accurate.

Secondly, an external replication of genetic associations is a rare endeavor. Unfortunately, as simply put by Kraft et al. (4), "*Genetic epidemiology learned the importance of replication the hard way.*" External validation studies perform at much lower rates, which underscores the significance of such efforts (5). Most genetic studies are still exploratory in nature, and false-positive results are inevitable. By prioritizing evidence-based targets, more resources can be allocated toward investigating variants with better promise of true impact on human health.

For UBC recurrence, the strongest result was mapped to *CWC27*. Previous study reported rs2042329 to correspond to higher expression of *CWC27* in bladder cancer cells (12). Additional functional analyses showed *CWC27* might affect bladder carcinogenesis via apoptosis. Interestingly, the original finding was made for Chinese patients, and authors failed to replicate the significance of rs2042329 on bladder cancer risk among Europeans (12). However, it is unknown if the lack of effect was also present for recurrence.

Additionally, it is surprising to see three SNPs in *NEIL2* being significant for NMIBC recurrence, especially keeping in mind the low likelihood of successful replication. Despite the high number of SNPs, strength of evidence for these associations is low, as reflected in Bayes Factor. Nonetheless, they might be promising targets in future replications. *NEIL2* is involved in DNA repair mechanisms, and research suggest it influences malignancies beyond bladder cancer. Alterations in normal *NEIL2* activity most likely result in accumulated oxidative damage, as elegantly presented by Benitez-Buelga et al. (19).

For UBC progression, the replicated variant maps to *NOS2*. The gene has been specifically linked to progression of various cancers (20, 21). It seems *NOS2* affects multiple oncogenic pathways that simultaneously affect tumor proliferation, angiogenesis, chemoresistance, and cell migration (20, 21).

As for UBC survival, three replicated SNPs are located in *RGS5*, *PCDC6*, and *EGFR*. Interestingly, a previous independent replication of SNPs associated with UBC prognosis has also successfully validated a variant in *RGS5* (rs12035879) for overall survival (OS) of MIBC cases (22). Comparison of two external replications offers potential insights—for example, the rs11585883 did not replicate in our study; however, another SNP in *RGS5* was successful, and associated with the same outcome (MIBC OS). These findings may be seen as cumulative toward the involvement of *RGS5* in cancer survival, even if specific SNPs are yet to be identified. We have checked if previously and current replicated *RGS5* SNPs are in LD, and they seem to represent independent signals in the gene ($R^2 = 0.03$ for rs12035879 and rs2344673 among Europeans). One major weakness of the replicated rs2344673 in our study is small sample size (29 cases and 109 controls). A *post-hoc* analysis on the overall survival of the whole sample, regardless of UBC invasiveness (209 cases and 1,325 controls) was not significant (data not shown). *RGS5* may not be relevant for all UBC patients, or might reflect power issues, which highlights further investigation being essential.

Remaining two genes implicated in UBC and NMIBC survival, namely *EGFR* and *PDCD6,* are both well-known cancer genes (14, 23). *PDCD6* seems to be heavily involved in apoptosis (14); however, the exact role of *PDCD6* is contrasting between various cancers (24), and further molecular research will help making evidence-based interpretations.

A replicated SNP (rs2293347) in *EGFR* has also previously corresponded to a protective effect on survival of lung cancer patients (25). The effect may be due to higher responsiveness to chemotherapy (26), which is a worthwhile investigation in future analyses.

Or study is subject to limitations, with one of the largest drawbacks being the difference between founders' and replication cohorts. A lot of studies have investigated populations of non-European ancestry, and it is possible we are not able to observe a true effect due to differences in LD of candidate SNPs in different samples. At the same time, the most reliable replication in our study was rs2042329, first reported in a Chinese population (12).

None of our replicated SNPs have passed the Bonferroni-corrected statistical significance level, suggesting some promising SNPs may have been identified by chance. Furthermore, current analyses have only focused on estimating the overall risk of a prognostic event, without considering the relevance of elapsed time to event. We see such and other more sophisticated analyses as a further direction in utilizing the described approach.

We were also unable to reliably estimate assigned treatment for UBC patients in the UK Biobank cohort, which would unquestionably confer to a more precise replication analysis. However, as the detail of released HES is increasing, we do not see this data out of reach and likely to include in future algorithm updates.

Finally, some replicated SNPs showed a conflicting direction of effect when compared to the original studies. These issues are likely to be clarified once more studies can confirm the overall association and establish the effect specifics.

To summarize, we have carried out an external replication of previously reported SNPs for UBC recurrence, progression, death and age using a novel approach of identifying clinically-relevant outcomes using HES data. Our analysis suggests specific targets, namely *CWC27*, *NEIL2*, *PDCD6*, *EGFR*, and *NOS2*, might be prioritized in efforts to further study the role of genetics in UBC prognosis. We are cautious about our findings, as there is no one metric or design to provide unquestionable evidence; instead, it should be viewed as one of the studies in a long line of accelerating research on UBC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NHS National Research Ethics Service North West (11/NW/0382). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NL designed the study, organized the data, performed statistical analyses, and wrote the first draft of the manuscript. All authors contributed to the manuscript and study design revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01082/full#supplementary-material

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REVIEW
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Urine Biopsy—Liquid Gold for Molecular Detection and Surveillance of Bladder Cancer

Uttam Satyal[1], *Abhishek Srivastava*[2] *and Philip H. Abbosh*[1,3]*

[1] Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA, United States, [2] Division of Urologic Oncology, Department of Surgical Oncology, Fox Chase Cancer Center, Philadelphia, PA, United States, [3] Department of Urology, Albert Einstein Medical Center, Elkins Park, PA, United States

With recent advancements in a non-invasive approach to cancer diagnosis and surveillance, the term "liquid biopsy" has gained traction but is currently limited by technological challenges in identifying and isolating circulating tumor cells (CTCs), proteins, cell-free DNA (cfDNA), or other nucleic acids. Tumor tissue biopsy, especially in genitourinary (GU) system is sometimes inadequate and requires invasive surgical options, especially for upper tract urothelial cancer. Urine can prove to be "liquid gold" since it may be a more abundant source of tumor-derived material without the background noise; however, urine DNA (uDNA) may be associated with low mutant allele fraction (MAF). Molecular detection of mutations in uDNA requires a sensitive and accurate method of analysis that allows a high depth of sequencing while minimizing artifacts. Several sequencing approaches to address this hurdle using enhanced library preparation techniques such as Tagged amplicon deep sequencing (TAm-Seq), Safe-SeqS, FAST-SeqS, and CAPP-Seq approaches have been developed. Urine biopsy utilizing next-generation sequencing (NGS) can prove useful at all stages of urologic malignancy care, where urine can be collected to aid in clinical decision making through the identification of commonly known mutations, and potentially reduce or avoid all forms of invasive procedures.

Keywords: urine biopsy, bladder cancer, cancer surveillance, prognosis and diagnosis, precision medicine, next generation sequencing

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Prashant Patel,
University of Birmingham,
United Kingdom

Reviewed by:
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Xin Xu,
Zhejiang University, China

***Correspondence:**
Philip H. Abbosh
philip.abbosh@fccc.edu

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INTRODUCTION TO LIQUID BIOPSY

Liquid biopsy refers to any non-tissue specimen, especially body fluids, that can be used to evaluate for tumors in the body, using any of several analytes such as circulating tumor cells (CTCs), proteins, cell-free DNA (cfDNA), or other nucleic acids present in the fluid. Liquid biopsy sources include blood, urine, other body fluids such as stool, saliva, pleural fluid, peritoneal fluid or washings, and cerebral spinal fluid (1), which can minimize the need for expensive, invasive, and sometimes painful tumor tissue biopsies to enable dynamic tumor monitoring. Cell-free tumor DNA or RNA extracted from liquid biopsies can potentially be used in a multiplicity of assays such as next-generation sequencing (NGS) or allele-specific PCR, etc. for the detection of mutations, translocations or copy number alterations, and the expression of specific markers of cancer at the mRNA/small RNA level. These alterations may be used as unique genetic signatures or single-gene tests (1, 2). Detection of somatic alterations and gene expression changes found in bladder tumors through the use of liquid biopsy of urine will be the focus of this review.

Blood is the most commonly described fluid used in liquid biopsy for many types of cancers (3, 4). Blood is the source of CTCs or circulating tumor DNA (ctDNA), circulating tumor RNA (ctRNA), and exosomes, released by tumor tissues, which can be potentially used to detect mutations present in the tumors. The major drawback of using blood as the source of ctDNA is that ctDNA comprises a tiny fraction of cell-free DNA present in the blood, which poses a significant obstacle for accurate and deep sequencing required to detect rare mutations. Moreover, cfDNA is always of low quality and fragmented to the approximate size of a nucleosome (140 bp), and ctDNA is variably present in the blood at earlier stages of cancer (5). So, alternate liquid biopsy approaches such as urine biopsy may be a richer source of tumor-derived material, especially for kidney, prostate, and upper and lower tract urothelial carcinoma, as urine bathes these genitourinary organs. Urine has other unique benefits such as ease of acquisition (does not require trained medical staff), lack of patient discomfort (increased patient compliance), and practically unlimited sample volume, and may have fewer contaminating proteins compared to blood.

Conventional diagnostic and biopsy modalities for bladder cancer include cystoscopy, ureteroscopy with or without biopsy, computed tomography (CT) scans with contrast, which are invasive, inadequate, and not without side effects (6, 7), but given the omnipresence of urine, there are surprisingly few effective liquid biopsy approaches that are widely used. Tavora et al. found that definitive diagnosis cannot be made because of the inadequate tissue in 25% of the renal pelvis or ureteral biopsies. Similarly, Gillan et al. reported significant under detection of carcinoma *in situ* (CIS) and discordance rate between the histopathology of biopsy and resected radical nephroureterectomy (RNU) specimens (7).

DIFFERENT COMPARTMENTS USED IN URINE BIOPSY

Urine can be used whole (i.e., "neat") or divided into two compartments useful for biomarker detection: supernatant and pellet. Supernatant consists of partially fragmented cell-free tumor nucleic acids and other tumor-derived materials, while the pellet primarily consists of exfoliated normal and cancer cells, as well as immune cells, debris, and possible bacteria. Several studies have shown that urine supernatant is superior to urine pellet for detection of genetic aberrations in urothelial cancer patients (8, 9). The cfDNA present in the urine supernatant may have higher mutant allele fraction (MAF), due to higher tumor turnover (necrosis/apoptosis) than DNA originating from exfoliated cells due to decreased contamination by normal urothelium and immune cells since those cells are not typically necrotic or apoptotic. Nevertheless, urine pellet has also been successfully used to detect mutations in the upper and lower tract urothelial carcinomas that matched with the mutation profile obtained from tumor tissues of respective patients (10, 11).

TECHNICAL CONSIDERATIONS IN URINARY DNA SEQUENCING

In order to detect very low MAF in urine DNA (uDNA), a sensitive and accurate method of analysis should be used that allows a high depth of sequencing while minimizing artifacts. NGS has the ability to detect rare mutations within a DNA sample but is relatively error-prone due to DNA polymerase errors and read errors during sequencing (12, 13). Although computational methods may identify and filter these variants, these methods are imperfect and may over-filter some true mutations. Use of barcodes or unique molecular identifiers before amplification can separate these errors form real mutation in uDNA (12, 14). It is currently unknown how low the MAF in urine will be, but one might reasonably expect it to potentially be very low after Transurethral Resection of a Bladder Tumor (TURBT), intravesical therapies, or systemic chemotherapy. For instance, prior work shows that there is a mean of 31 mutant copies with a mean of 2018 total copies per mL of urine in patients with bladder cancer recurrence (2). This translates to an *average* MAF of 0.015; many mutations will be present at lower MAF. Although this is low and presents a significant challenge, the problem is even worse in the plasma ctDNA environment.

Several sequencing approaches address this obstacle using enhanced library preparation techniques. Tagged amplicon deep sequencing (TAm-Seq)-based NGS utilizes efficient library preparation and statistical analysis to detect mutations across a gene panel with a detection limit of 0.02% and specificity of 99.99% (15, 16). The Safe-SeqS approach tags each template DNA with unique molecular identifiers prior to amplification to create a unique family of sister molecules descended from the same original molecule resulting in reliable detection of 0.1% MAF with a specificity of 98.9% (12, 17). FAST-SeqS can detect mutation using degenerate bases at 5′ end of the primer that is used as a molecular barcode to label each DNA template (18). CAPP-Seq is an approach that sequences recurrently mutated exons that can detect mutation with allele frequency down to 0.02% with 93% specificity (19). This technique was further improved with unique duplex molecular identifiers and additional informatics filtering to detect mutation allele frequencies as low as 0.004% and specificity of 99.99% (20).

Methods besides NGS are available for liquid or urine biopsy. Droplet digital PCR (ddPCR) and mass spectrometry methods can also be used to detect somatic variants. Droplet digital PCR is based on a water-in-oil emulsion where the tumor or normal DNA is distributed into millions of droplets followed by amplification using TaqMan fluorescence probes which are specific to either the mutant or normal sequences (21). Because the DNA is in limited concentration at the time of droplet formation, droplets tend to either have only one mutant or only one WT allele (or no allele), such that when the template is amplified within the droplet, there is an unambiguous mutant or WT readout within that droplet. This greatly enhances the sensitivity of the method when droplets are sorted by color. The sensitivity of 93% with 100% specificity, with an allele frequency detection limit of 1 in 100,000 molecules have been reported (21).

URINE BIOPSY IN UROLOGIC MALIGNANCY SURVEILLANCE

Urine has direct contact with bladder tumors, enabling the possibility of relatively large tumor marker quantities (22). Urine can be collected at several diagnostic stages to aid in clinical decision making: prior to presentation as a screening tool; at the time of workup of microhematuria, gross hematuria, or urinary symptoms suggestive of urothelial carcinoma; as a marker of residual disease after treatment; or as a marker of recurrence of urothelial carcinoma (2, 23).

Somatic hotspot mutations within the promoter region of *TERT* are one of the most frequently occurring mutations in different cancers including bladder cancer, of which the most common variants are C>T transition at either of two positions: chr5:12952228 and chr5:1295250, 146 and 124 base-pairs upstream, respectively, of start codon (10, 24, 25). The high frequency of *TERT* promoter mutation has been shown to be prevalent in both muscle-invasive and non-muscle invasive bladder cancer and can be easily detected in urine (10, 25). Kinde et al. analyzed uDNA from 76 patients with non-invasive urothelial carcinoma and showed that mutation in the *TERT* promoter region could be used as a biomarker for early detection of disease in patients being worked up for bladder cancer (11). In addition, they showed that analysis of urinary DNA *TERT* promoter hotspots after TURBT could be used as a marker for recurrent urothelial carcinoma. In another study, *TERT* promoter mutation was significantly associated with 6-month recurrence of pT1 bladder cancer presence of *TERT* mutation increased the risk of recurrence 5-fold, and *TERT* promoter hotspots could be used to non-invasively follow up non-muscle invasive bladder cancer patients after surgery (26). However, these studies were conducted in a small number of patients at a single center and superiority over urine cytology and surveillance cystoscopy still needs to be established for widespread utilization (11, 26).

Similarly, *FGFR3* is mutated in two-thirds of non-muscle invasive bladder cancers [at one of 5 hotspots, with S249C being by far the most common (27)], and the detection of *FGFR3* mutation in urine biopsy was associated with 4-fold higher risk of recurrence (28, 29). In another study, mutation of *FGFR3*, *RAS,* and/or *PIK3CA* hotspots were analyzed using urine biopsy. At least one of these mutations was present in about 90% of the recurrences, making it feasible to predict the onset of recurrence prior to clinical manifestations (30). Reliance on *FGFR3* mutations is ideal for low grade disease, as these variants are common for these cancers (31). However, the argument can be made that these are the least clinically impactful tumors. Although they recur frequently, almost never progress. Improved biomarkers for low grade/low stage disease are probably not necessary.

Patel et al. showed that the presence of mutations detected by either targeted hotspot panel or copy number alteration detected with shallow whole genome sequencing in uDNA during second neoadjuvant chemotherapy (NAC) cycle was associated with recurrence of bladder cancer with 83% sensitivity and 100% specificity, while the persons without mutation had low recurrence rate with 100% positive predictive value and 85.7% negative predictive value (22). They also revealed that uDNA could be analyzed to assess the tumor evolution during NAC of urothelial carcinoma. This is a highly provocative study but may be impractical to incorporate clinically given that whole genome sequencing was required to detect genetic aberrations in the tumor for 1/3 of the patients.

Several urine biomarkers of urothelial malignancies are FDA-approved for detection and surveillance, five of which use protein-based assays, while UroVysion™ is the only that uses genetic markers (32). UroVysion™ uses exfoliated urothelial cells from urine and analyzes chromosome aneuploidy along with loss of locus 9p21 for the detection of recurrent bladder cancer (33, 34). Meta-analysis showed a sensitivity of 72% and specificity of 83%, with better performance in high-grade urothelial carcinoma, but ∼40% sensitivity in low-grade urothelial carcinoma (35, 36). Positive Urovysion™ test in BCG treated patients with superficial bladder cancer was related to treatment failure and high risk of progression to muscle-invasive bladder cancer (37, 38). In comparison, it seems likely that NGS-based methods to detect genetic alterations will be much more sensitive.

The role of non-coding RNAs in bladder cancer has recently emerged in the diagnosis and prognosis of bladder cancer. Two types of non-coding RNAs have been described- small non-coding RNA and long non-coding RNA (lncRNA). The mature forms of these non-coding RNAs act as regulators of gene expression and are never translated into proteins. Micro-RNAs (miRNAs) are an example of a small non-coding RNA subclass that has been investigated extensively. Several studies have reported downregulated or upregulated miRNAs in bladder cancer (39–41). lncRNAs have also been associated with bladder cancer development and progression, although their overall expression and functional significance is still uncertain (42, 43). An essential difference between lncRNAs and miRNAs is their size, with lncRNAs having more than 200 nucleotides. Yazarlou et al. detected the expression levels of four lncRNAs (LINC00355, UCA1–203, UCA1–201, and MALAT1) in urinary exosomes and found that three of them were highly expressed in patients with bladder cancer (44). The combined diagnostic model of lncRNA showed a higher sensitivity (92%) and a higher specificity (91.7%) compared with traditional biomarkers. Seitz et al. identified novel lncRNAs in bladder cancer that act as oncogenic drivers contributing to an aggressive cancerous phenotype through interaction with proteins involved in the initiation of translation and/or post-transcriptional modification of RNA (42, 45).

URINE MOLECULAR BIOMARKERS FOR PRECISION MEDICINE

It is important to distinguish how and where urine biopsy could potentially be applied clinically. Prognostic biomarkers such as those described for surveillance are biomarkers that associate with long-term outcome/prognosis, i.e., residual disease status or clinical stage. Predictive biomarkers are associated with or deterministic of response to a particular therapy. Urine

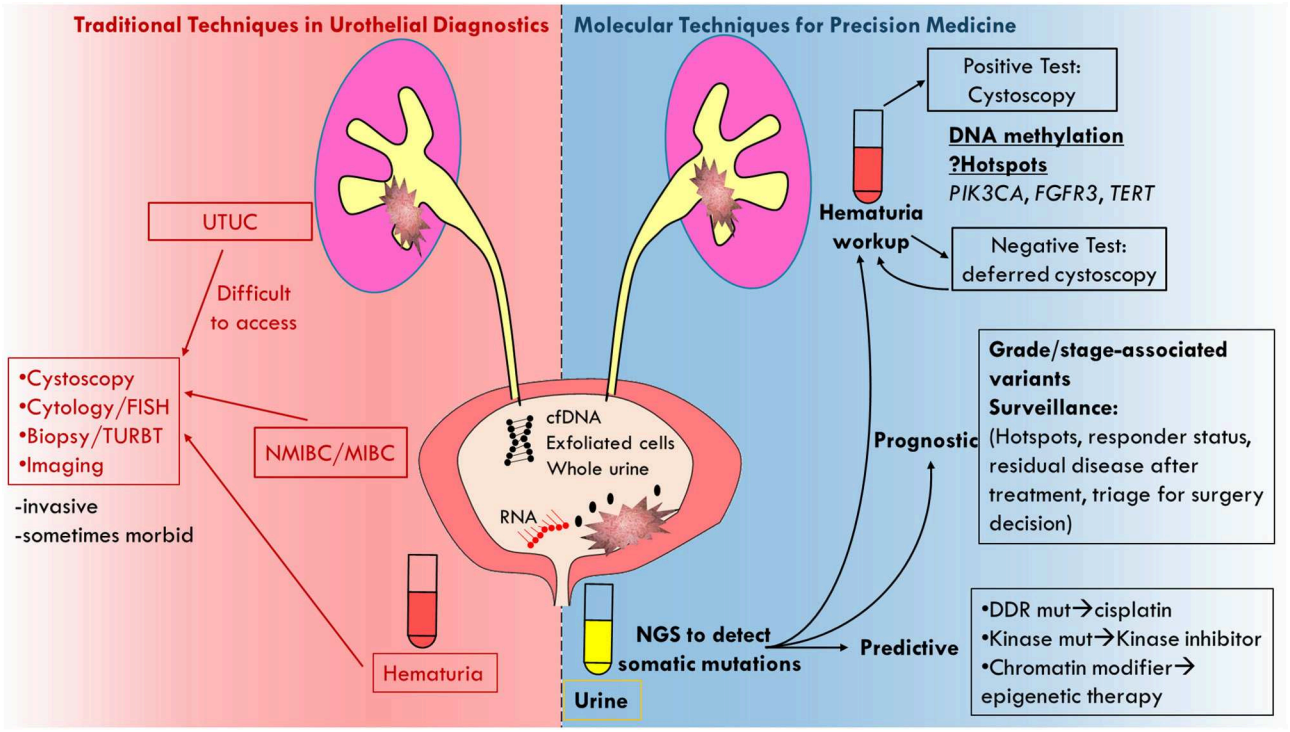

FIGURE 1 | Cartoon depicting molecular analysis of urine biopsy as superior technique to traditional techniques in urothelial diagnostics.

biopsy may potentially be used in both settings as we describe below (**Figure 1**).

Prognostic Urine Biopsy Applications

Urine biopsy has the potential to be used in monitoring disease response and/or resistance to any therapy that is used for the treatment of bladder cancer including TURBT, intravesical BCG/chemotherapy, systemic chemotherapy or immunotherapy, and radiation. The highest utility of prognostic urine biopsy is probably in the curative/localized setting (46, 47). In particular, urine biopsy could be used to aid in clinical decision-making surrounding cystoscopy in the workup of hematuria (i.e., the initial diagnosis of bladder cancer), follow-up cystoscopy for bladder cancer surveillance, TURBT/bladder biopsy and potentially even radical cystectomy, avoiding unnecessary cost and complications.

Initial Workup of Hematuria

American Urological Association (AUA) guidelines recommend performing cystoscopy in patients presenting with gross or microscopic hematuria [with only 3–28% of hematuria patients being diagnosed with bladder cancer depending upon other risk factors such as smoking history, prolonged exposure to chemicals and/or radiation (48)]. Even in patients with risk factors, many negative cystoscopies will be performed to identify a single case, resulting in high diagnostic cost and significant patient burden (49, 50). The safe avoidance of invasive testing/cystoscopy is a desirable outcome for patients and might be achieved with urine biopsy, where test-negative patients could either avoid cystoscopy or undergo deferred cystoscopy. Van Kessel et al. measured DNA methylation in urine biopsy samples and used multivariable analysis of clinical risk factors in hematuria patients to achieve 93% sensitivity and 86% specificity for bladder cancer, thus potentially reducing the need for diagnostic cystoscopy by 77% (50). A limitation of this study was that information on microscopic vs. macroscopic hematuria and cytology were not available and clearly modify the risk of a positive test and of the diagnosis of bladder cancer.

Cxbladder® is urine-based assay that uses reverse transcription quantitative PCR (RT-qPCR) to amplify and detect mRNA level of *CDK1*, *HOXA13*, *MDK*, *IGFBP5*, and *CXCR2* for the detection and surveillance of bladder cancer (51). Cxbladder® *Detect* uses these genotypic factors to detect bladder cancer in hematuria patients with a sensitivity of 82% and specificity of 85% (52). Cxbladder® *Triage*, on the other hand, uses these genotypic factors along with phenotypic factors such as age, gender, frequency of macrohematuria and smoking history to rule out bladder cancer in hematuria patients and have achieved sensitivity as high as 95% and negative predictive value of 97% (53, 54).

UroSEEK®, a massively parallel sequencing-based assay developed by Springer et al., which detects mutations in *FGFR3*, *TP53*, *CDKN2A*, *ERBB2*, *HRAS*, *KRAS*, *PIK3CA*, *MET*, *VHL*, and *MLL*, promoter region of *TERT*, and detection of aneuploidy. It has been shown to be effective for the detection of urothelial carcinoma, including bladder and upper tract urothelial cancer

TABLE 1 | Initial evaluation for patients presenting with microscopic hematuria or dysuria using the uroSEEK test (55).

	Bladder cancer $n = 177$	No bladder cancer $n = 393$
UroSEEK positive	147	**28**
UroSEEK negative	**30**	365

The bold values indicate the number of patients diagnosed incorrectly (false negative or false positive) using respective methods.

TABLE 2 | Initial evaluation for patients presenting with hematuria using the Cx bladder triage and detect test (57).

	Bladder cancer $n = 45$	No bladder cancer $n = 391$
Cx bladder triage and detect positive	38	**78**
Cx bladder triage and detect negative	**7**	313

The bold values indicate the number of patients diagnosed incorrectly (false negative or false positive) using respective methods.

(55). UroSEEK was able to detect 83% of bladder cancer cases, which increased to 95% when coupled with cytology, while the sensitivity among upper urothelial carcinoma patients was 75%. Another recent study from Stanford University showed that a high-throughput sequencing-based hybrid capture method for urine tumor DNA detection, uCAPP-Seq, could detect bladder cancer with 84% sensitivity and 96–100% specificity (56).

If these tests were applied to a clinical setting, patients being worked up for the diagnosis of bladder cancer who have positive urine prognostic DNA methylation or mRNA detection tests would be further subjected to cystoscopy, while test-negative patients might be placed into a cystoscopy deferral program. UroSEEK and Cxbladder offer increased sensitivity especially when combined with urine cytology, however; in patients presenting with microscopic hematuria, UroSEEK and Cxbladder missed 30/177 = 16.9% and 7/45 = 15.6% patients with bladder cancer, respectively (**Tables 1, 2**) (55, 57). Although cystoscopy is an uncomfortable test for patients to undergo, missing a clinically significant bladder cancer which would have been detected cystoscopically is a high diagnostic bar to overcome.

Bladder Cancer Surveillance

Recurrences occur in up to 50% of non-muscle invasive bladder cancer patients depending on the stage, multifocality, size, and grade of the tumor, and this necessitates lifelong surveillance cystoscopy in high-risk cases (58, 59), which makes bladder cancer the most expensive cancer that is treated in America (30, 56). Use of urine biopsy in follow up can potentially improve quality of life by reducing the need for invasive testing. Urine biopsy could foreseeably result in cost reduction too, if such a test had good long-term prognostic power (i.e., a "one and done" test), although this has not been rigorously borne out yet. Dudley et al. used urine CAPP-Seq technique to detect mutations in uDNA for the surveillance of bladder cancer after intravesical treatment, being able to detect recurrent cases in overall 91% of patients that included all patients with positive cytology and more than 80% of the patients that cytology missed (56). Kinde et al. analyzed DNA from urine cell pellets using Safe-SeqS technique in the aforementioned study to show that the presence of *TERT* promoter mutation in uDNA can be directly correlated with recurrence (11). As previously mentioned, the detection of *FGFR3*, *RAS,* and/or *PIK3CA* mutations can also predict recurrence with excellent accuracy (22, 27–30).

Cxbladder®*Monitor* is commercially available that can be used to test recurrent urothelial carcinoma by detecting mRNA level of five urine mRNA biomarkers *IGF*, *HOXA*, *MDK*, *CDC*, and *IL8R* gene expression along with few clinical variables. The overall sensitivity of 91% and a negative predictive value of 96% within 95% CI was observed for this assay and had reduced sensitivity of 86% for low-grade Ta (54, 60).

Cost-effectiveness of such strategies would only be achieved if the cost of testing all patients to avoid cystoscopy in most patients would cost less than performing cystoscopy in all patients in the absence of a test. Other factors would also need to be considered, such as the cost of missing a diagnosis, the cost of working-up a patient with a false-positive result, and potential complications avoided from invasive procedures. These additional costs will vary depending on whether the missed tumor is a high-risk or low-risk superficial bladder cancer or muscle-invasive. Besides, the benefit of a urine biopsy in this clinical scenario would ostensibly be earlier detection of a recurrent tumor, leading to earlier treatment. Early detection of a low-grade recurrence is not likely to bend the clinical destiny of bladder cancer patients, but early detection of a high-risk recurrence might be more meaningful if it resulted in treatment prior to progression to a muscle-invasive state. Therefore, urine biopsy in surveillance might optimally be applied to patients with higher-risk urothelial cancers. It is important to note that a bladder cancer screening test for asymptomatic patients, or even in high-risk populations such as smokers, would be very difficult to effectively achieve given the low incidence of bladder cancer on a population-based scale.

Enhanced Diagnosis of Abnormal Bladder Lesions

BCG is well-known to induce inflammatory changes in the urothelium, and often these can be mistaken for CIS or other malignant manifestations, prompting biopsy of suspicious lesions which merely harbor benign inflammatory changes. Urine biopsy could potentially provide an extra diagnostic dimension to triage these abnormal lesions into groups of those meriting biopsy or treatment under anesthesia vs. those which can be observed.

Decisions Regarding Radical Cystectomy

NAC is associated with a 30–40% ypT0 rate at the time of radical cystectomy (61–63). There is a significant desire among patients and urologists to avoid radical cystectomy in patients who achieve ypT0 after NAC due to the morbidity, cost, and complications associated with this disease. Clinical assessment of residual disease status after NAC is challenging with high local recurrence rates in patients achieving cT0 states. Meyer et al. in their study reported 28% relapse rate for muscle-invasive

disease and 24% relapse rate for non-muscle invasive disease after achieving cT0 status following NAC (64). Similar results have been reported by several multi-institutional studies (65, 66). Therefore, clinical T0 assessment is not equivalent to a pathologic assessment of a ypT0 state (i.e., in a surgical specimen). Urine biomarkers might enhance the accuracy of the staging of residual disease after NAC by detecting small amounts of tumor genetic material for enhanced staging in order to better identify complete responders for cystectomy avoidance algorithms.

Predictive Urine Biopsy Applications

In addition, urine biopsy might be used as a predictive biomarker similarly to what has been described using ctDNA for lung cancer or other cancers. For instance, ctDNA can be used to identify *EGFR* mutations for treatment assignment to EGFR inhibitors, and similarly can be used to identify the emergence of resistance to these drugs (67). As kinase inhibitors gain traction in the treatment of urothelial carcinoma (68), these agents will likely be applied in earlier settings, and urine biopsy might be used to guide treatment decisions or detect the onset of resistance mechanisms. Afatinib, an irreversible inhibitor of the EGFR family of kinases, was shown to be effective only in platinum-refractory metastatic urothelial carcinoma patients with *ERBB2* and *ERBB3* gene alterations (69). One might envision the use of afatinib in patients with localized cancers whose tumors contain mutations in *ERBB2* or *ERBB3*, which are common in muscle-invasive bladder cancer (70). This could be given in a biomarker-selected and neoadjuvant fashion, whereby patients are selected based on urine biopsy or tissue-based genetic tests. Given the preponderance of FGFR3 alterations in bladder cancer, FGFR3 inhibitors in biomarker-selected patients using a urine biopsy might be a highly desirable path forward.

Additionally, alterations in DNA repair genes are associated with the increased response of bladder cancer patients to NAC and chemoradiation (71–73). These could foreseeably be detected by urine biopsy and used to triage patients into NAC as well.

CHALLENGES AND FUTURE PERSPECTIVES

Urine is "liquid gold" for prognosis, diagnosis, and monitoring of tumor evolution after NAC, BCG treatment, or radiotherapy, especially in patients with upper tract urothelial carcinoma where anatomical considerations make accurate staging challenging. Work in this area will continue to evolve and improve until clinical testing is a reality. Urinary biomarkers are low-hanging fruit in the genomics age and given the absence of widely used biomarkers in urothelial cancers, they would fill a significant need for patient evaluations.

Significant challenges need to be considered though. As mentioned, the low MAF is only the first. Tumor DNA present in urine is prone to degradation in the absence of proper storage and transportation from clinic to molecular biology laboratory. Urine biopsy will require new technologies to preserve the integrity and fidelity of these samples. Fixation of tissue introduces well-known artifacts in NGS analyses (74), and this would ideally be avoided in urine biopsy diagnostic media. False positives or negatives will affect the diagnosis, prognosis, and surveillance of urothelial cancer if urine biopsy from the patients is not stored or transported correctly. Although novel devices for collection, storage, and shipment of urine cell pellets have been described (75), little work has been done to identify novel hi-fidelity fixation methods. Immediate processing and frozen storage would likely preserve the integrity and fidelity of DNA in the sample, but this processing method would be challenging for most centers which may not have immediate access to such equipment such as a centrifuge or −80°C freezer. Although advances in sequencing technology and informatics have made sequencing for detection of tumor DNA more feasible and practical, it is still expensive (i.e., not cost-effective) for serial monitoring of tumor evolution after therapy. Moreover, if it were cost-effective, it may not always be clear what a clinician would do with a positive surveillance test with the absence of clinical manifestations—a change in therapy might be needed, but a change to what? It is not clear yet if it would be safe to avoid cystoscopy or cystectomy, for instance. These questions would need to be answered in prospective trials in order to make meaningful and safe changes to the care of biomarker-selected patients.

Should a consensus panel of genes to be sequenced for urothelial carcinoma be used? It may depend on the clinical question. One might envision a predictive test to focus on currently druggable targets, whereas a prognostic test might be a better test if it included non-druggable targets in order to increase the sensitivity of the test. It will be necessary to develop tests that address specific clinical questions in an accurate, precise, and unambiguous manner. Urine biopsy will likely continue to evolve toward higher specificity and sensitivity along with (hopefully) the reduction of associated costs, adding compliance and comfort to patients suspected of having bladder cancer and establishing itself as an integral part of urology or urologic oncology clinics. However, again, it is critical to maintain the development of urine biopsy tests that address a specific and genuine need in the management of urothelial cancer. The market is littered with high performing tests that never gained traction because they did not address a specific clinical need (or at least do not address it unambiguously), were too expensive, or only add an incremental amount of information to the clinical decision-making process. We believe that urine biopsy utilizing NGS-based methods has the potential to significantly enhance clinical decision making for urothelial cancer patients and their care providers in urology, oncology, and pathology.

AUTHOR CONTRIBUTIONS

US and AS wrote the manuscript. PA revised and edited the manuscript.

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REVIEW
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Immune Responses in Bladder Cancer-Role of Immune Cell Populations, Prognostic Factors and Therapeutic Implications

Magdalene Joseph[1]* *and Deborah Enting*[2]

[1] Hayday Laboratory, School of Immunology and Microbial Sciences, King's College London, London, United Kingdom,
[2] Department of Uro-Oncology, Guy's Hospital, Guy's St Thomas NHS Foundation Trust, London, United Kingdom

Immunosurveillance, which describes the immunologically mediated elimination of transformed cells, has been widely accepted in the context of bladder cancer for many decades with the successful use of Bacillus-Calmette Guerin for superficial bladder cancer since the 1970s. With the emergence of checkpoint inhibitor blockade in the treatment of urothelial cancers, there has been a resurgent interest in the immunology of bladder cancer. The theory of cancer immunoediting proposes that the immune system has both pro-tumorigenic and anti-tumor effects, the balance between the two determining the progression of an individual tumor. However, whilst there is evidence for the action of various immune cell populations in bladder cancer, a cohesive picture of the immune response to bladder cancer and its driving forces are still lacking. Additionally, little is still known about the normal immune landscape of the bladder. Future progress in bladder cancer therapeutic approaches will require a strong foundation in understanding the immunology of this disease. This review considers the evidence for the role of the main immune cell populations, both innate and adaptive, in the immune response to bladder cancer. Recent research and overarching themes in the immune response to bladder cancer are explored. The minimal evidence regarding the normal immune landscape of the human bladder is also summarized to contextualize downstream immune responses. Of specific interest are the innate and myeloid populations, some of which are resident in the human bladder and which have significant effects on downstream adaptive tumor immunity. We discuss factors which restrain the efficacy of populations known to have anti-tumor activity such as cytotoxic T cells, including the constraints on checkpoint blockade. Additionally, the effects on the immune response of tumor intrinsic factors such as the genomic subtype of bladder cancer and the effect of common therapies such as chemotherapy and intravesical Bacillus Calmette-Guerin are considered. A significant theme is the polarization of immune responses within the tumor by a heavily immunosuppressive tumor microenvironment which affects the phenotype of multiple innate and adaptive populations. Throughout, clinical implications are discussed with suggestions for future research directions and therapeutic targeting.

Keywords: immunosurveillance, bladder cancer, genomic subtypes, Bacillus Calmette-Guerin, tumor microenvironment

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Edited by:
Kent William Mouw,
Dana–Farber Cancer Institute,
United States

Reviewed by:
Vadim S. Koshkin,
University of California San Francisco,
United States
Randy Sweis,
University of Chicago, United States

***Correspondence:**
Magdalene Joseph
magdalene.joseph@kcl.ac.uk

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INTRODUCTION

Tumor immunosurveillance describes the ability of the immune system to recognize and eliminate transformed cells early in the tumorigenic process. By this definition, clinically detected cancers usually represent a failure of host tumor immunosurveillance. Whilst controversial when first proposed by Paul Erlich in the early 1900s, there is now a vast body of experimental and observational evidence suggesting an active role for the immune system in eliminating transformed cells (1, 2). More recently, growing awareness of some of the pro-tumorigenic actions of specific immune cell populations has led to a more comprehensive theory of cancer "immunoediting" (2).

Immunoediting describes how pro- and anti-tumorigenic responses of the immune system, in concert with the properties of the tumor itself, can alter the clinical course of a tumor through "selecting" for less immunogenic clones. This theory proposes three stages in the evolution of a tumor- "elimination" (which if successful would abort the tumorigenic process); "equilibrium" where the tumor begins to gain immune-evasive properties that enable its survival; and "escape" whereby it overwhelms the immune system's defenses and is usually fatal unless treated (**Figure 1**). It is in the "equilibrium" and "escape" stages that tumors usually become clinically apparent, when some immunoevasive features have already developed. The older term "immunosurveillance" usually refers to the "elimination" part of this process. Little is known about proposed downstream immunoediting but evidence for immunosurveillance in bladder cancer is longstanding (3, 4).

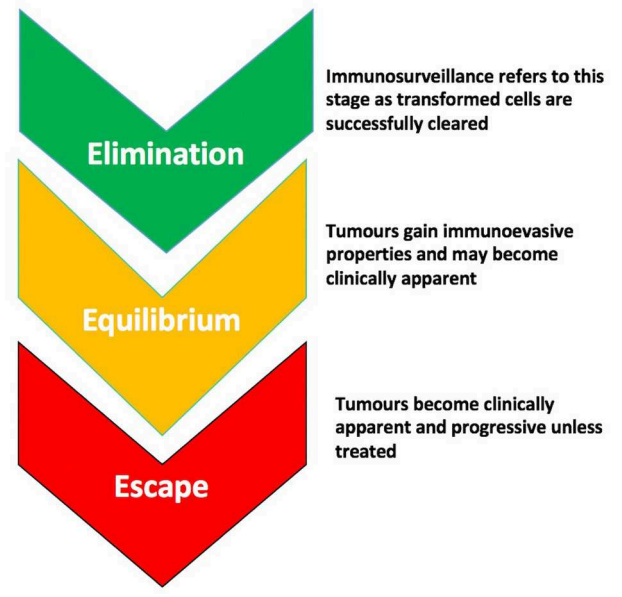

FIGURE 1 | The stages of immunoediting. There are 3 stages proposed in the evolution of a tumor- "Elimination," which if successful, leads to the abortion of the tumorigenic process through immunological mechanisms; "Equilibrium" during which the tumor gains immunoevasive properties allowing it to circumvent or suppress the anti-tumor effects of immune cells; and "Escape" during which the tumor overwhelms immune defenses and is usually progressive and fatal unless treated.

In fact, a role for immunosurveillance in bladder cancer has been tacitly accepted since the 1970s when Morales et al. first demonstrated reduced tumor recurrence after intravesical Bacillus Calmette-Guerin (BCG) therapy (5). BCG treatment is now widely used in the treatment of superficial bladder cancer and arguably the most successful immunotherapy in use. However, the mechanisms underlying the anti-tumor response triggered by BCG and the general features of the immune response in bladder cancer are incompletely understood to this day. The advent of immune checkpoint-directed therapy makes it imperative to understand the mechanisms of immunosurveillance and immunoediting; and identify predictive immunological biomarkers for treatment and outcome.

Candidates for such biomarkers have included tumor infiltrating lymphocytes (TILs) and tumor somatic mutational burden (TMB). It has become clear over the past two decades that some cancers are more infiltrated by lymphocytes which appears to have prognostic significance in certain cancer settings (6, 7). It had been postulated that high lymphocyte infiltration may be secondary to a high TMB, and thus greater neo-antigen load. However, the evidence in bladder cancer suggests that it may be mutations in specific pathways rather than the quantity of somatic mutations which underlies immune infiltration (8). Additionally, there has been increasing interest in the innate and myeloid populations which are found abundantly in all tumors and are implicated in suppressing tumor immunosurveillance. Thus the search is on for more sophisticated biomarkers which integrate recent advances in tumor immunology.

In this review, we will explore what is known of the resident immune populations of the bladder. We will consider the role of both resident and recruited immune cell populations in the immune response to bladder cancer, focussing on their prognostic significance and the potential to therapeutically manipulate each population. We will also review the relevance to immune responses of recent advances in genomic and molecular subtyping and the effect of therapies as varied as chemotherapy, BCG and checkpoint blockade.

THE IMMUNE LANDSCAPE OF THE BLADDER IN HEALTH

There are few studies which have explored the bladder immune landscape in health and much of this knowledge comes from six mainly immunohistochemical analyses of bladder mucosa undertaken from the 1980s onward.

These have used biopsies from small cohorts (5–13 subjects) of healthy control subjects or patients with non-bladder related pathologies- most recently, subjects were brain-dead, ventilated organ donors. There is no quantification of the different immune subsets in naïve human bladder, however, a picture can be built from the different qualitative studies (**Figure 2** and **Table 1**). The earliest analyses suggest the urothelium is largely populated by HLA-DR +ve cells, some of which appear to be morphologically dendritic cells and some of which stain for the Langerhans'

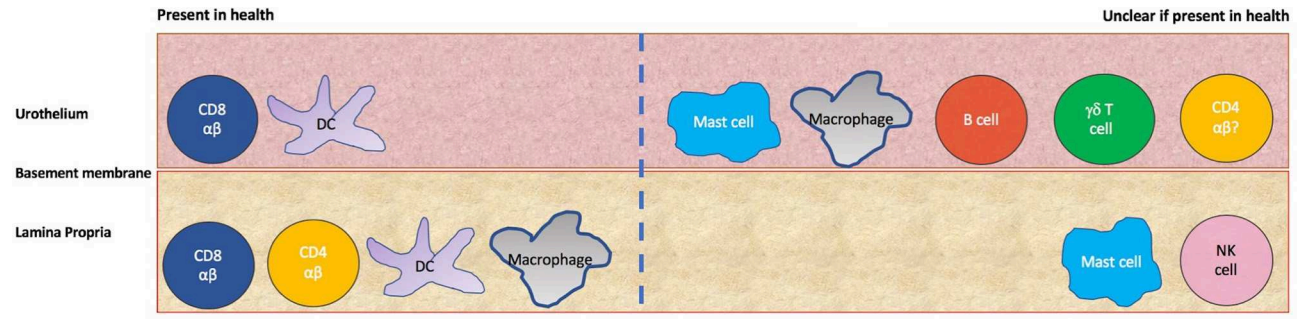

FIGURE 2 | Immune populations in the human urothelium and lamina propria. On the left side are those populations whose presence is confirmed by more than one study, whilst on the right are those populations where uncertainty exists about tissue residence in health due to a lack of evidence or contradictory evidence from different studies.

TABLE 1 | Evidence for immune populations resident in naïve human and mouse bladder.

Population of interest	Human urothelium (four studies)	Human lamina propria (single study)	Mouse whole bladder (single study)
Neutrophils	Not described	Not described	Absent
B cells	Seen in 25% of cohort in one study, absent in smaller study previously	B cells not seen	B cells not examined
γδ T cells	Absent in earlier smaller study but seen in 55% of cohort in newer study	γδ T cells absent	γδ T cells present
NK cells	NK cells absent	Seen very occasionally (but used CD57 which is less specific marker)	NK cells present
CD4 T cells	Seen in 70% cohort in newer larger cohort but absent in 2 earlier studies	CD4 T cells present	CD4 T cells present
CD8 T	CD8 T cells present in all studies	CD8 T cells present	CD8 T cells absent
Dendritic cells	Seen in 3 studies, 2 of which found Langerhans type dendritic cells	Present with some Langerhans type dendritic cells	Dendritic cells present
Macrophages	Seen in newer cohort but absent in 2 older studies	Macrophages present	Macrophages and monocytes present
Mast cells	Mast cells seen in only study which investigated this	Mast cells seen	Mast cells and eosinophils seen

A summary of the evidence for residence of different immune cell populations in the bladder. Evidence is presented separately for human urothelium, human lamina propria and whole mouse bladder. Evidence is derived from 6 studies published between 1986 and 2001 which are as follows: Mora-Bau et al. (9); Christmas et al. (10, 11); Gardiner et al. (12); Saint et al. (13); Cresswell et al. (14).

marker Cd1a, suggestive of Langerhans' like dendritic cells (15, 16). There is a smaller population of resident urothelial CD8 +ve T cells and immune cells in the urothelium were found to largely reside next to the basement membrane (10, 15, 16). In the lamina propria were more HLA-DR +ve cells, some with a macrophage-like appearance (15, 16). CD8 T cells and occasional CD4 T cells were also present in this layer (10, 15). Mast cells were found to reside in both layers using a toluidine dye staining method (11).

Whilst the earlier studies found an absence of γδ T cells, macrophages and CD4 T cells within the urothelium itself (10, 15, 16), the most recent analysis in 2001 found all three present in the urothelium in most donors with another small study finding CD3+ve CD8–ve T lymphocytes in the urothelium which could correspond to CD4 and γδ T cells (13, 14). Additionally, CD57+ve cells along the basement membrane which were characterized as natural killer (NK) cells in an earlier study were absent in the most recent study which used the same marker, now known not to be specific to NK cells (10, 15, 16). Whether these differences between earlier and later series are due to technological advances in immunohistochemistry, increased sample size or the nature of the donor cohorts is unclear.

The immune landscape in naïve mouse bladder is characterized more quantitatively by a single recent study (9). Though the method of whole bladder digest and flow cytometry does not allow for delineating the urothelium and lamina propria, it is a much higher sensitivity technique than those used for normal human bladder immunophenotyping. This showed that around 70% of CD45+ve cells in murine bladder are antigen presenting cells (APC). Macrophages constituted the largest APC population forming around 40% of CD45+ve cells, with dendritic cells a further 20% of CD45+ve cells. The rest was composed of a mix of CD4 T cells, NK 1.1+ve NK cells, γδ T cells and cKit+ve mast cells (9).

Interestingly, the CD8 T cells which are so prominent in naïve human bladder across multiple studies were completely absent in naïve mouse bladder suggesting that adaptive functions

may be performed by γδ T cells instead which constituted around 2% of CD45+ve cells in the bladder (9). This potentially fundamental difference between murine and human bladder in health highlights the limitations of translating murine model findings to human disease.

However, this emerging picture of which populations normally reside in the bladder is essential for understanding downstream responses to bladder carcinogenesis. We will now consider the role of specific immune populations and their functional behavior in bladder cancer, with a focus on implications for prognosis and therapy.

THE ROLE OF IMMUNE CELL POPULATIONS IN BLADDER CANCER IMMUNOSURVEILLANCE

Some key immune cell populations such as dendritic cells are already resident in the human bladder (7, 14, 15) and additional numbers of these can be recruited from the circulation. However, others such as neutrophils, FoxP3+ve regulatory T cells (T regs) and myeloid derived suppressor cells (MDSCs) are entirely recruited from the circulation in response to factors secreted by the tumor itself or surrounding immune cells. We will begin by considering cells of the innate immune system. These cells, which encompass some lymphoid populations, are still little understood or manipulated therapeutically despite being crucial gatekeepers and modulators of the immune response to cancer. Alongside sections on individual populations are sections discussing some of the most significant therapies and areas of research in bladder cancer and their relevance to the immune response in bladder cancer. These discussions include the relevance of genomic subtyping of bladder cancer and the impact of chemotherapy, BCG and checkpoint inhibition on immune responses.

MACROPHAGES AND THE POLARIZING EFFECTS OF THE TUMOR MICROENVIRONMENT

Macrophages have been detected consistently in immunohistochemical studies of healthy human bladder (13, 15, 16) and appear to have an overall pro-tumorigenic role. Multiple studies have correlated high tumor associated macrophage (TAM) counts with poor survival and poor response to treatment including chemotherapy and BCG therapy (17–19).

In vitro, macrophages are known to exhibit certain anti-tumor functions including phagocytosis, release of reactive oxygen species and secretion of inflammatory cytokines (20, 21). However, it is clear that these anti-tumor functions are largely lost in most cancers and TAMs have been found to be polarized to an immunosuppressive "M2" phenotype in multiple cancers

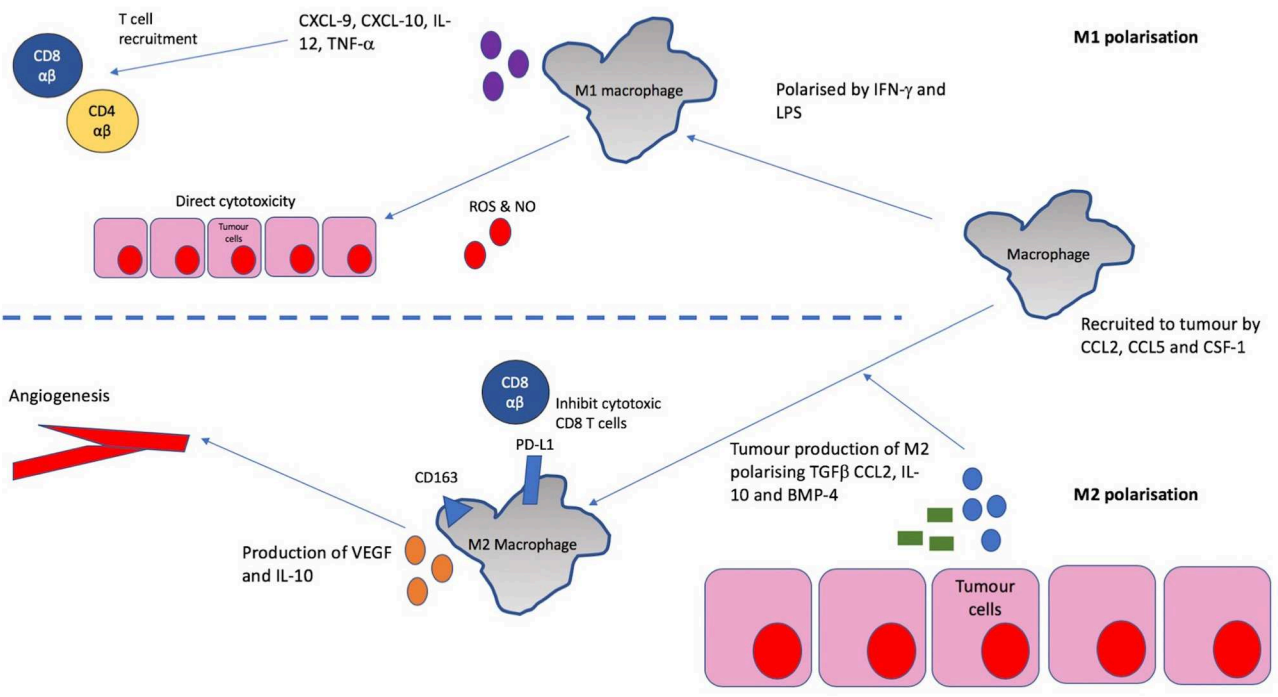

FIGURE 3 | Recruitment and M2 polarization of macrophages in bladder cancer. Macrophages are recruited to the tumor by chemokines such as CCL2, CCL5, and CSF-1 which are often released by the tumor itself. In the tumor, they are polarized toward an M2 phenotype by the action of mediators including TGF-β, CCL2, IL-10, and BMP-4. This phenotype is characterized by CD163 expression, suppression of T cell responses, tissue remodeling and promotion of angiogenesis. This is mediated amongst other things by the production of vascular endothelial growth factor (VEGF) and IL-10, and the high expression of inhibitory checkpoint molecules such as PD-L1.

(**Figure 3**) (22, 23). This is associated with CD163 expression, IL-10 production, angiogenesis and tissue remodeling in murine models of various cancers- all of which can contribute to increased tumor growth and metastasis (24). In fact, one study in human bladder cancer found that an increasing CD163/CD68 ratio (and therefore, increased M2 polarization as CD68 is a pan-macrophage marker) correlates with higher disease stage and vascularity (25) suggesting M2 polarized macrophages are responsible for the angiogenesis seen in bladder tumors.

Whilst, IFN-γ and LPS are capable of polarizing macrophages to a "M1" pro-inflammatory phenotype associated with cytotoxicity (24), these are rarely the predominant players in the tumor microenvironment. Production of M2 polarizing cytokines such as CCL2, IL-10, and TGF-β by bladder tumors has been demonstrated in various *in vitro* studies (26–28) and IL-10 production by bladder tumor cells has been shown to induce an immunosuppressive monocyte phenotype (**Figure 3**) (29). There may also be a role for bone morphogenic proteins (BMPs) produced by bladder tumors in M2 polarization, with a recent study finding BMP-4 induces a M2 macrophage phenotype in bladder cancer *in vitro* (30).

In addition to their effects on tissue remodeling and tumor angiogenesis, M2 macrophages promote tumorigenesis partly through their effects on the adaptive immune system in their function as antigen presenting cells (APCs). It has been demonstrated in co-culture experiments that IL-10 production by bladder cancer cells leads to increased PD-L1 expression on monocytes and downstream suppression of T cell immune responses (29). Additionally, M2 macrophages lack production of chemokines such as CXCL9 and CXCL10 which recruit Th1 lymphocytes with anti-tumor activity (23). This may explain findings in a cohort of 296 patients where the strongest association with poor survival was predicted by a high CD68/CD3 ratio (31) suggesting that macrophage high tumors may correlate with poor T cell infiltration.

In fact, a recent study categorized tumors on the basis of two stromal immune infiltration patterns and found that the subtype with low macrophage infiltration and high cytotoxic lymphocyte infiltration was associated with improved survival with the presence of these populations inversely correlated (17). Thus, whilst macrophages do not directly influence clonal selection in tumors and immunoediting, they appear to broadly suppress adaptive immunosurveillance and create a tumor favoring microenvironment in bladder cancer. Any therapeutic strategy which aims to improve on current response rates, has to address this key axis of immunosuppression.

GENOMIC SUBTYPES OF BLADDER CANCER AND IMMUNOSURVEILLANCE IMPLICATIONS

Also greatly affecting immune cell infiltration into tumors is the intrinsic genomic subtype of bladder cancer which affects prognosis as well as response to therapies (32). The genomic subtype is often a reflection of the layer or tissue of origin of the tumor. Multiple sub-classifications have been proposed over the years based on different cohorts of patients and a recent attempt to reach a consensus has identified 6 main subtypes in muscle invasive bladder cancer, some of which are more immune cell infiltrated than others (33). Basal/squamous tumors, the commonest subtype (∼35%), arise from the basal layer of the urothelium and are enriched for mutations in tumor suppressors such as p53 and RB1 (33).

Despite being heavily infiltrated with immune cells, including cytotoxic T cells and NK cells expressing high levels of inhibitory checkpoint receptors, these tumors do not respond to immunotherapy as well as less heavily infiltrated tumors (33). This suggests that the local tumor environment might be too immunosuppressive to overcome with single agent immunotherapy alone. A recent study analysing immune subset infiltration in bladder cancer using bulk transcriptomes (CIBERSORT) found that M2 macrophage infiltration was associated with the basal subtype of bladder cancers and a higher histological and pathological grade suggesting that M2 macrophages may be responsible for the poor response to immunotherapy seen in this group and thus a target for future intervention (34).

On the other end of the spectrum, the luminal unstable subtype, which arises from the luminal layers of the urothelium and is the subtype with the highest mutational load, does not demonstrate any associations with an immune infiltrating signature (33), despite the possibility of more neoantigens within the tumor. However, this subtype shows greater benefit from checkpoint inhibitor immunotherapy than the heavily immune infiltrated basal squamous subtype (33). These findings suggest that susceptibility to immune therapies does not correlate directly with mutational load or depend simply on the baseline level of immune infiltration (33). It is clear one has to consider the interplay between the tumor and multiple immune populations which are capable of polarization toward pro- or anti-tumorigenic actions.

Neutrophils are one such population which demonstrate similar pro-tumorigenic polarization to macrophages in most disease settings. They are of particular interest in the context of BCG therapy where this usual pro-tumorigenic polarization appears to be reversed by an external intervention highlighting the importance of immune modulating therapies.

NEUTROPHILS, THE NEUTROPHIL-LYMPHOCYTE RATIO AND BCG IMMUNOTHERAPY

Neutrophils are absent in healthy bladder, but are found in higher proportions in the circulation in cancer patients and abundantly in bladder tumor where they appear to have a largely immunosuppressive effect unless their activity is modulated by concomitant therapies (35). Numerous studies have examined circulating neutrophil-lymphocyte ratio (NLR) as a possible biomarker to predict prognosis or response to treatment in bladder cancer. A systematic review in 2016 analyzed NLR in urothelial cancer, covering 23 studies and 6,240 patients (36). It found that a high NLR appeared to correlate with worse overall,

recurrence-free and cancer specific survival (36) with similar findings in a meta-analysis of NLR as a prognostic marker in non-muscle invasive bladder cancer (37). It has also been shown that higher tumor infiltrating neutrophil count and NLR both predict advanced pathological stage and poorer survival confirming the link between what is seen in the circulation and the tumor milieu (38).

One of the mechanisms by which this circulating neutrophilia and accumulation in tumor develops is likely related to the direct release from tumor cells of cytokines such as CXCL1, CXCL5 and, in particular, IL-8 which is known to be a potent chemoattractant for neutrophils. IL-8 is known to be constitutively produced by urothelium in health (39) but many human bladder cancer cell lines overexpress the cytokine (40, 41). In humans, urinary levels of IL-8 have been shown to correlate with the presence of transitional cell carcinoma and its stage (42) and circulating levels of IL-6 and IL-8 have been correlated to NLR in a cohort of 121 patients (43).

Evidence from non-bladder cancer mouse models suggests that the local cytokine milieu in the tumor has a significant effect on neutrophils, with TGF-β polarizing toward an immunosuppressive phenotype (35, 44). As bladder cancer is known to produce higher levels of TGF-β than normal tissue (28), this might be a mechanism by which tumor infiltrating neutrophils are polarized toward immunosuppression and explain the correlation between tumor infiltrating neutrophils and poor survival. These immunosuppressive "N2" polarized neutrophils, which express high levels of arginase (45), are known to assist invasion, metastasis and angiogenesis in other cancers through the release of proteases such as neutrophil elastase and matrix metalloproteinase-9 (MMP-9) (35). *In vitro* experiments using "N2" neutrophils polarized by co-culturing with bladder cancer cell lines demonstrated that these neutrophils enhanced the invasiveness of the bladder cancer cells in "*in vitro*" assays (46).

However, tumor infiltrating neutrophils can take on a completely different role following therapeutic manipulation or a change in the tumor environment. Blocking the effects of TGF-β with an orally administered small molecule inhibitor in a mouse tumor model led to the accumulation of neutrophils with a cytotoxic and anti-tumor phenotype compared to those in control mice (44). In fact, the actions of neutrophils can be essential in some settings.

BCG therapy for superficial bladder cancer is one of the earliest prototypic immunotherapies where neutrophils are essential to the anti-tumor effect (47, 48). Often used to treat superficial non-muscle invasive bladder cancers (NMIBC), BCG is used at a stage where the tumor burden, and thus TGF-β levels and any adversely polarizing effects on neutrophils, is minimal. The uptake of mycobacterium by the urothelium and resident antigen presenting cells triggers release of cytokines such as IL-6, IL-8, GM-CSF, and TNFs which induce a rapid and heavy infiltration of neutrophils into the bladder within hours of instillation in patients undergoing BCG therapy (48, 49).

In fact, mice depleted of neutrophils show no therapeutic benefit from BCG when compared to untreated controls, with an absence of CD4 T cell influx (47) which is known to be essential to the anti-tumor response. The mechanism of action in murine models appears to depend on indirect recruitment of T cells through the recruitment and activation of monocytes, mediated through the release of cytokines such as CXCL1, IL-8, MIP-1, and MIF (47). Thus, it appears that neutrophils mediate the influx of later immune mediators of the anti-tumor BCG response such as monocytes and CD4 T cells (**Figure 4**). In addition, there is emerging evidence of direct anti-tumor effects through the release of apoptotic mediators such as tumor necrosis factor-related apoptosis- inducing ligand (TRAIL) which is known to be selectively cytotoxic to tumor cells (50). It has been shown that urinary excretion of TRAIL positively correlates with response to BCG in humans, with TRAIL demonstrating direct cytotoxicity of bladder tumor cells *in vitro* (51).

Additionally, there is *in vitro* evidence that BCG activated macrophages exhibit pro-inflammatory and tumoricidal properties, demonstrating the favorable effects of this therapy on multiple innate populations (21). It is clear from the evidence considered that triggering innate immune responses can have clinically significant effects on downstream adaptive immunity. Such repolarization of usually immunosuppressive populations toward anti-tumor responses raises the tantalizing prospect of future therapies targeting innate populations.

MYELOID DERIVED SUPPRESSOR CELLS AND THE IMMUNOLOGICAL EFFECTS OF CHEMOTHERAPY

Closely related to monocytes and neutrophil precursors, myeloid derived suppressor cells are immature myeloid cells which show immunosuppressive properties and are absent in healthy individuals. It is thought that the chronic inflammatory milieu of cancer induces the release of immature myeloid cells from the bone marrow and they are often found amongst the mononuclear cell fraction in peripheral blood and in tumors of various types (52). The granulocytic subtype, G-MDSC, identified as CD11b+veCD14–veCD15+ve are the predominant subtype found in malignant settings with a minor monocytic subtype, M-MDSC, which is CD11b+veCD14+veHLA–DR–ve/lowCD15–ve (52). CD33, a marker of immature myeloid cells can additionally be used to characterize these cells with G-MDSC expressing low levels and M-MDSC expressing high levels. The main barrier to characterizing the role of these cells has been the lack of consistency across studies in the way these cells are identified using surface markers (53). However, there is ample evidence that immature myeloid cells have adverse effects in bladder cancer.

High numbers of peripheral blood MDSC were found to adversely correlate with stage, grade and prognosis in bladder cancer by Yang et al. in 2016, though the specific subtypes of MDSC were not analyzed (54). Previously, Eruslanov et al. had shown that G-MDSCs were expanded in the PBMC fraction of bladder cancer patients compared to healthy individuals, whilst there was no similar expansion of the M-MDSC subtype (55). These G-MDSCs were capable of significant pro-inflammatory cytokine production and inhibited *in vitro* T cell proliferation by inducing FoxP3+ve regulatory T cells (55).

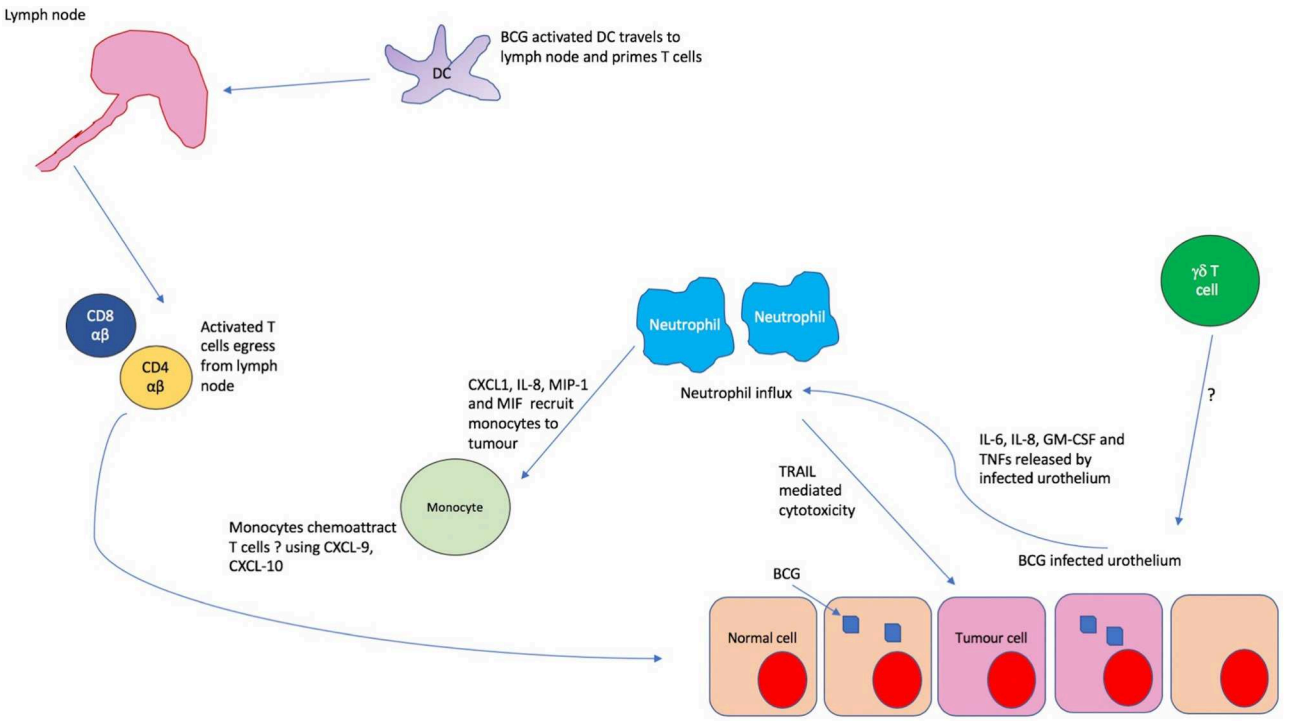

FIGURE 4 | The immunological cascade in BCG treatment. Uptake of BCG by bladder urothelium triggers release of IL-6, IL-8, GM-CSF and tumor necrosis factors (TNFs) which recruit copious neutrophils to the bladder. These neutrophils chemoattract monocytes to the tumor environment through the release of CXCL1, IL-8, MIP-1, and MIF. It is thought that these monocytes subsequently induce T cell migration, possibly through the release of chemokines such as CXCL9 and 10. In the meantime, BCG activated dendritic cells migrate to the lymph nodes and prime antigen naïve T cells which then egress from the lymph node and are recruited to the tumor. Whilst γδ T cells are found in large numbers in the urine post BCG, it is unclear how they are recruited to the bladder.

These patterns are further reflected in the bladder tissue itself where G-MDSC were the predominant MDSC subtype present in an analysis of fresh bladder tissue (56). The degree of infiltration of G-MDSC negatively correlated with the CD8 T cell infiltration in another study (57), in keeping with the T cell suppressive effects observed previously by Eruslanov et al. The MDSC present in bladder tumor have been shown to express high levels of immunosuppressive molecules such as Arginase 1, inducible nitric oxide synthases (iNOS) and PD-L1 and directly suppress T-cell proliferation reflecting their phenotype in the peripheral blood (58).

Whilst, we have mostly considered G-MDSC, a role for M-MDSC in the context of response to BCG has been suggested by Chevalier et al. who found a T-cell to MDSC ratio of <1 correlating with a poorer recurrence free survival post treatment (59). Recruitment of these cells may represent one of the factors underlying BCG failure in some patients.

Interestingly, treating PBMCs derived from patients with bladder cancer with cisplatin has been shown to selectively deplete G-MDSC (57). Additionally, T cells cultured with cisplatin-treated peripheral blood G-MDSCs exhibited less inhibition of their tumor apoptosis promoting capabilities than those cultured with untreated G-MDSCs (57). This suggests one of the anti-tumor effects of cisplatin may be mediated by reshaping the immune compartments in bladder cancer and suppressing G-MDSC proliferation and function. Immune enhancing properties of chemotherapy have been observed in other cancers and this is an exciting area for further research (60).

NATURAL KILLER CELL SUBSETS AND γδ T CELLS

Thus far we have considered the innate, myeloid immune populations and their usually pro-tumorigenic role in bladder cancer, in the absence of additional therapies. Bridging the divide between the innate and adaptive immune system are lymphoid cells of the innate immune system and unconventional T cells such as natural killer cells (NK) and γδ T cells. These are thought to be one of the earliest effectors of the anti-tumor response and do not depend on MHC-restricted antigen presentation for activation (61). These cells are capable of recognizing stress ligands which are overexpressed by tumor cells, including MICA, MICB and the ULBP family, and respond with perforin-mediated cytotoxicity or IFN-γ production (2, 61). IFN-γ is capable of polarizing other immune populations, including macrophages, toward a type 1 anti-tumor response.

Evidence for the role of natural killer (NK) cells in bladder cancer is patchy and inconclusive at best and they do not appear to be a resident population in healthy human bladder (10, 62).

However, a retrospective study of patients with non-muscle invasive bladder cancer (NMIBC) found that baseline NK cell infiltration was significantly higher in the group of patients who had recurred at 2 years follow up compared with disease free patients (63). There was also an association between tumor size and increased NK cell infiltration (63) which appears to suggest lack of efficacy, or worse, an adverse role.

Surprisingly, a more recent flow cytometric analysis of fresh tissue from patients with both NMIBC and muscle invasive bladder cancer (MIBC) has suggested that improved survival may be linked to the presence of $CD56^{bright}$ NK cells within the tumor which are found in much smaller numbers than their $CD56^{dim}$ counterparts. These cells are functionally active compared to the reduced cytokine secreting capacities of the $CD56^{dim}$ subset (64). This might explain the findings of the previous study which did not distinguish between the subsets in their analysis.

NK cells are also of particular interest in the context of BCG where a lack of efficacy of BCG in NK-cell deficient beige mice has been reported (65). However, it appears the presence of monocytes is essential, at least for the $CD56^{dim}$ population. Bisiaux et al. investigated the dependence on monocytes for activation by BCG and found that γδ T cells and $CD56^{bright}$ NK cells appeared to be capable of activation by BCG alone, in the absence of monocytes, whereas αβ T cells and $CD56^{dim}$ NK cell activation was dependent on monocytes (66). Thus it appears that there are two NK cell populations with very different behaviors and associations in bladder cancer and these will have to be studied individually in future analyses.

NK cell mediated cytotoxicity is dependent on receptors such as NKG2D which bind stress ligands that are overexpressed on tumor cells. Experiments blocking NKG2D show reduced cytotoxicity of NK cells against bladder cancer lines demonstrating the importance of this mechanism of tumor recognition (67) and these receptors are highly expressed on other cytotoxic populations including CD8 αβ T cells and NKT cells.

One lymphocyte population bearing high levels of NKG2D and of particular interest in the context of BCG are γδ T cells which are a resident population in naïve mouse bladder and may be resident in healthy human bladder (9, 62). These cells bear a rearranged γδ TCR but are capable of non-MHC restricted activation, cytotoxicity and cytokine production. Multiple studies have shown that this otherwise small lymphocyte population is enriched in the urine and tissue of patients undergoing BCG therapy for bladder cancer and the level of increase seems to correlate with a positive outcome from therapy (68, 69). This has been further bolstered by mouse studies which have shown a lack of effect of BCG in γδ deficient mice, which appears to be dependent on their production of IL-17 and recruitment of neutrophils (70). Given that γδ T cells can be activated by BCG in a monocyte-independent fashion as discussed earlier, this positions them center stage in the response to BCG treatment.

Whilst γδ T cells can also be activated by NKG2D ligands, another possible mode of activation in this context is through the Vγ9δ2 TCR which is activated in a phosphoantigen dependent manner, with phosphoantigens being abundant in BCG. In support of this hypothesis, γδ T cells produced in urine post-BCG therapy were enriched for this subset in one study (69). Promisingly, a survival benefit from intravesical administration of this subset of human γδ T cells has been demonstrated in a murine orthoptic bladder cancer model (71).

The role of $CD56^{bright}$ NK cells and γδ T cells outwith of BCG therapy remains unclear but the evidence for their independent activation by BCG raises the possibility of enhancing BCG therapy by using the potentiating effects of these cells. γδ T cells are currently being trialed as an adoptive therapy in various cancers and may yet enter the realms of bladder cancer therapy.

We will now consider the responses of the adaptive immune system, beginning with the classical gatekeepers to an adaptive immune response, dendritic cells.

DENDRITIC CELLS

Dendritic cells (DC) are potent mediators of adaptive immunity through their ability to present antigen to and activate T cells. They constitute around 20% of CD45+ve cells in the naïve mouse bladder and dendritic appearing HLA-DR +ve cells have been identified in healthy human bladder suggesting residence (9, 15, 16). These cells constantly sample their environment for antigens and, in the context of danger signals such as heat shock proteins released from necrotic tumor cells, they can be activated and migrate to the lymph nodes where they prime naïve T cells (20).

Whilst a relatively understudied area in bladder cancer, there is evidence that dendritic cell number or function may be affected in the context of bladder cancer. Few studies, mainly examining myeloid subset DCs, have shown that peripheral blood dendritic cell counts appear to be reduced in patients with bladder cancer compared to healthy individuals (72, 73). One study found that surgery increases this peripheral blood DC count relative to baseline in those with superficial disease (74). This suggests that dendritic cells may be depleted from the blood by the tumorigenic process.

In support of some depletive mechanism would be the observation that high levels of tumor infiltrating DCs in human bladder cancer predict progression to muscle invasion suggesting that DCs may be a significant but unhelpful presence in bladder cancer (75). Dendritic cells have been shown to be a significant part of the tumor infiltrate, constituting around 17% of CD45+ve cells within the tumor in one study (76). These DCs appeared phenotypically immature with low CD80 and CD86 expression and this immaturity persisted even in higher stage tumors that had greater numbers of infiltrating DCs (76).

This blocking of maturation was further confirmed by Beatty et al. who found immature DCs in the tumor and urine of patients with superficial disease pre-BCG (77). All of this suggests a functional deficit in dendritic cells in the context of bladder cancer and experiments have shown that monocyte derived dendritic cells adopt an immature phenotype when co-cultured with bladder cancer cells (78, 79).

Malignancy associated glycan, Sialyn-Tn, has been implicated in this process and shown to induce an immature phenotype in human monocyte derived dendritic cells *in vitro* (79). These low HLA-DR, CD80, and CD86 DCs were impaired in their

ability to activate T cells. The T cells were, in fact, skewed toward a FoxP3-high, IFN-γ- low regulatory phenotype. This skewing of the immune response and blocking of DC maturation was partially reversed by blocking of Sialyn-Tn antigens, CD44 and MUC1, suggesting a causal relationship between specific glycan expression by tumors and inhibition of DC maturation (79).

However, there are likely to be a multitude of inhibitory influences on DCs in the tumor microenvironment and the upregulation of the JAK2/STAT3 pathway in tumors has been implicated in blocking DC maturation *in vitro* (78). An inhibitor of JAK2, AG490, was found to partially reverse maturation block of DCs providing a possible therapeutic target (78).

Whilst recent approaches to overcoming such blocks have focused on genetically modified DCs or *ex-vivo* antigen loading and activation, these have had modest effects *in vitro* and have not translated into significant clinical outcomes. Lapuleucel-T, a dendritic cell based therapy using monocytes activated with recombinant GM-CSF linked to a HER2 peptide showed no statistically significant overall survival benefit or disease free survival benefit in a cohort of high risk HER2+ve bladder cancer patients in a phase 2 study (80, 81).

However, there might be a role for DCs in potentiating BCG therapy. In a small cohort of 12 patients undergoing BCG therapy for superficial disease, Beatty et al. found that the 6 patients who responded to BCG had a trend toward increased urinary DCs post treatment with the reverse in the non-responding group (77). A role for DCs in the response to BCG is further suggested by a study which found that myeloid DC numbers in the blood and urine rose in response to BCG treatment (72).

Given the previous evidence of maturation block of DCs in bladder cancer, this suggestion of a positive effect of DCs on clinical outcome might be explained by findings that BCG induced maturation of peripheral monocyte derived DCs which were then able to activate natural killer T cells (NKT) and γδ T cells to lyse bladder cancer cells (82). Thus, BCG may be capable of converting the immunosuppressed, immature DC phenotype to an anti-tumor one. In fact, when BCG was added to co-cultures of PBMCs and bladder cancer cells, minimal inhibition of tumor growth was observed unless BCG-infected DCs were added (83).

Thus, like macrophages and neutrophils, the anti-tumor capabilities of dendritic cells too appear largely suppressed by the tumor microenvironment with BCG possibly providing a way to reverse this inhibitory effect. It appears that the tumor is capable of steering these key myeloid populations toward actions which globally suppress immunosurveillance of the tumor. As we will see in the next section, such immunosuppressive polarization also affects the lymphocyte populations of the adaptive immune system.

CD4 HELPER T CELLS AND THE Th1/Th2 AXIS

CD4 T cells are resident in the murine bladder and may constitute a resident population in healthy human bladder (9, 10, 15, 62). However, they are most potently recruited to the immune response through encountering activated dendritic cells in the lymph nodes (84). On encountering their cognate TCR ligand on an activated DC, naïve CD4 T cells can acquire different phenotypes which depend on the cytokine milieu and phenotype of the dendritic cell. Murine and human *in vitro* experiments, as well as mouse models have established that a Th1 phenotype is acquired in the presence of IL-12 ± IFN-α production and is characterized by high IL-2 and IFN-γ production and tumor suppressing responses (84, 85). On migration to the tumor milieu, such Th1 polarized cells can promote macrophage and CD8 T cell mediated cytotoxicity through IFN-γ production.

The significance of Th1 cells to the anti-tumor response is highlighted by a recent study investigating the effect of checkpoint blockade in a murine bladder cancer model. Analysis of the immune infiltrates from tumor and lymph nodes post treatment revealed an expansion of IFN-γ producing CD4 T cells (Th1) and the neutralization of IFN-γ abolished the anti-tumor effect of checkpoint blockade suggesting the key role for these cells and this cytokine (86). Additionally, a Th1 biased response is known to be essential to successful BCG therapy (87, 88) with a recent study demonstrating a transient but significant recruitment of CD4 helper T cells to the bladder in a murine model- recruitment which far outstripped that of cytotoxic CD8 or regulatory FoxP3 T cells (89).

In contrast, Th2 polarization which is characterized by the promotion of humoral immunity requires IL-4, the source of which is less clear, and is characterized by IL-4, IL-10, and IL-13 production, the net effect of which is to suppress cytotoxic immune responses (84, 85). CD4 T cells are additionally capable of adopting a regulatory phenotype (Treg) characterized by FoxP3 and CD25 expression or an IL-17 producing Th17 phenotype (90, 91). However, as these are often induced peripherally in the context of cancer, we will consider these in a separate section.

No less important a role for CD4 T cells is their reciprocally activating effects on dendritic cells through the CD40L-CD40 axis, further potentiating the antigen presenting capabilities of DCs and promoting cytotoxic lymphocyte activation (84). Whilst CD4 T cells are detected in bladder tumors, little is known about their functional potential and many studies are limited by the lack of functional phenotyping of the different subclasses.

A higher CD4 T cell density within the tumor was found to correlate with a poor prognosis in a study of 131 patients with NMIBC and, suggesting a similar adverse correlation for CD4 T cells, a high CD3/CD4 ratio was found to correlate with better survival in MIBC in an analysis of 4 publicly available genomic datasets (92, 93). However, both of these studies are limited by the aforementioned lack of functional phenotyping of the CD4 T cells present.

A recent study of methylation patterns of CD4 T cells from the tumor in patients with bladder cancer showed that a higher stage was correlated with increased methylation (and thus reduced expression) at the IFN-γ locus (94). Conversely, patients with a complete response to neoadjuvant chemotherapy showed significant hypomethylation at loci related to all functional types, but most prominently at the IFN-γ locus confirming the anti-tumor role expected of Th1 polarized cells (94).

However, the picture in untreated or progressive bladder cancer is one of dominant Th2 polarization with Satyam et al. finding IFN-γ and IL-2 levels to be significantly lower in the blood of patients with bladder compared to healthy individuals with the inverse true of Th2 cytokines IL-4, IL5, and IL-10 (95). Intriguingly, a role in Th2 polarization has been suggested for the little known double positive CD4+veCD8+ve T cell population which was found to be expanded in the blood of patients with bladder cancer in a recent study. These cells were shown to be capable Th2 cytokine production and inducing Th2 polarization in naïve CD4 T cells (96).

Whilst suggestive of an adverse correlation with Th2 skewing, the evidence above is inconclusive for the role of Th1 and Th2 helper CD4 cells in bladder cancer. Similarly ill-defined is the role of the CD4+ve FoxP3+veCD25+ve regulatory T cell which we will consider next.

FoxP3 REGULATORY CELLS AND Th17 HELPER T CELLS

Regulatory FoxP3+veCD25+ve CD4 T cells (Tregs) are known to have significant tumor promoting effects in many cancers and can be of thymic origin or induced locally through the actions of TGFβ in concert with other immunosuppressive cytokines such as IL-10 (97, 98). In fact, blocking IL-10 and TGFβ has been shown to reduce the induction of CD25+ve regulatory T cells *in vitro* by a bladder cancer cell line (26). Such Tregs are capable of suppressing immune responses to cancer, often through their suppression of cytotoxic T cells and constitute a significant percentage of TILs in bladder cancer- a median of 17% across samples in one study (99). However, a clear role for regulatory T cells in bladder cancer is unclear with some studies suggesting a positive correlation with clinical outcomes.

Winerdal et al. used immunohistochemistry to analyse 37 cystectomy specimens ranging from pT1 to pT4 disease and found a higher infiltration of FoxP3 cells correlated with improved survival (100). They suggested this may be secondary to FoxP3 being upregulated on T cell activation, thus acting as a marker of activated T cells, rather than regulatory T cells alone. However, in support of a true anti-tumorigenic role, the same group found an inverse correlation between "true" Tregs at the invasive front of urothelial cancers and the expression of matrix metalloproteinase 2 (MMP2) which is produced by tumor cells and macrophages and promotes tumor invasion (101). Within their cohort, which included all T stages of disease, presence of Tregs at the invasive front also correlated positively with survival. The group found that these Tregs bore epigenetic marks of Treg differentiation and stably expressed FoxP3 suggesting they are "true" Tregs (101).

However, numerous studies have found negative correlations between FoxP3 T regulatory cells and survival including one study of 115 cases of NMIBC which found an inverse correlation between FoxP3 cell frequency within the tumor (as % of CD3+ve cells) and recurrence-free survival (99). Additionally, higher FoxP3 infiltration in the stroma around the tumor was shown to predict a shorter recurrence free survival post-BCG treatment for NMIBC (102). In the context of MIBC, a higher CD8/FoxP3 tumor infiltrating lymphocyte TIL ratio predicted better response to neoadjuvant chemotherapy though the density of either considered alone did not show this same correlation, with another study finding the same correlation with CD8/FoxP3 TIL ratio and survival post cystectomy (103, 104).

It is difficult to reconcile these very contradictory findings which show different prognostic associations for Tregs. This may be due to variation in the location of the Tregs being measured- whether within the tumor or surrounding the tumor. It is also possible that low level FoxP3 or CD25 expression by activated non-regulatory T cells may cloud the picture when additional makers are not used to fully phenotype regulatory T cells.

An additional question surrounds the antigen specificity of possible suppressive responses by Tregs, with a recent murine study suggesting that suppression of naïve CD4 responses by Tregs is antigen specific and related to removal of peptide-MHC II complexes from the surface of dendritic cells (105). Whilst no evidence exists for this mechanism of Treg mediated suppression at present in humans, it might explain how immunoediting operates through antigen specific immunosuppression to select for specific tumor clones.

One important cell type to consider in concert with regulatory T cells is the pro-inflammatory Th17 subgroup of CD4 T helper cells which are characterized by the expression of the RORγt transcription factor. Unlike, Tregs which can have a thymic origin, these cells are exclusively induced in the periphery and their differentiation appears to be reciprocally regulated with respect to Tregs. In humans, IL-1β and IL-6 are the key cytokines implicated in their induction (90). Th17 cells have been found to be enriched in bladder tumor relative to peripheral blood from patients and healthy individuals, suggesting a local induction of this sub-group by bladder tumor (90).

However, the role of Th17 cells in bladder cancer is still largely unstudied. In mice, responses to BCG have been shown to depend on IL-17 production, albeit by γδ T cells (70). However, in the absence of additional therapies, IL-17 may be pro-tumorigenic through its angiogenic effects amongst others and IL-17 knockout mice exhibit reduced growth of orthoptic bladder and melanoma tumors (106).

Thus, the role and prognostic significance of Tregs and Th17 cells in bladder cancer remains unclear. One population where there is no doubt about their prognostic significance is CD8+ve cytotoxic T cells. The evidence overwhelmingly suggests a favorable correlation with outcomes and their antigen specificity implies a degree of immunoediting and we will discuss these in this final section.

CYTOTOXIC T CELLS AND THE PROMISE OF CHECKPOINT INHIBITOR IMMUNOTHERAPY

Cytotoxic CD8 T cells have been the focus of immuno-oncology for the past decade with the success of checkpoint inhibition in multiple cancers, including bladder cancer. Studies suggest they are a resident population in human bladder and they are present

in high densities in some molecular subtypes of bladder cancer (9, 10, 33, 62). CD8 T cells in the tumor are believed to be specific for tumor associated antigens and a recent analysis of the TCRβ repertoire (both CD4 and CD8 T cells) in MIBC correlated low TCR diversity and high neoantigen load with improved survival suggesting that tumor antigen specific immune responses are a key part of anti-tumor immunity (107).

Whilst some studies have suggested a favorable prognostic significance for the presence of CD8 T cells in bladder cancer (108), others have suggested that this association appears to be modulated by the presence of other immune cell populations, including regulatory FoxP3+ve T cells. As previously mentioned, a favorable response to neoadjuvant chemotherapy was found to be predicted by a high CD8/FoxP3 ratio in the tumor, but not the level of infiltration of either population alone (103).

We have discussed earlier the suppression of T cells responses by expression of PD-L1 on macrophages and monocytes within the tumor. The significance of this pathway is strengthened by studies demonstrating that CD8 T cells infiltrating bladder tumors often show high expression of PD-1 (109, 110), the receptor for PD-L1, which is known to be characteristic of an exhausted phenotype in the context of chronic antigenic stimulation. This chronic antigen exposure may result from the inhibition of their cytotoxic potential by tumor cells with one study showing that urothelial cancer supernatants suppressed perforin expression in CD8 T cells. This was associated with upregulation of TGFβ signaling pathways, suggesting that this is TGF-β mediated (111). Additionally, the immunosuppressive environment of the tumor with a lack of IFN-γ and IL-12 production and production of immunosuppressive cytokines by multiple immune populations can directly suppress CD8 T cell responses (2).

The exhausted phenotype of CD8 T cells has been correlated with a shorter recurrence free survival in a recent study which examined PD-1 expression on urine derived lymphocytes pre-cystectomy (112). However, this exhaustion can be exploited therapeutically and the PD-1/PD-L1 axis in bladder cancer has been targeted with checkpoint inhibitor blockade with some success over the past 5 years. Overall, response rates range from 15 to 50% dependent on patient selection and biomarker enrichment (113). Despite these findings, the prognostic and predictive value of expression of PD-1 or PD-L1 within the tumor remain incompletely defined (114).

There is also the untested possibility that CD8 T cell mediated cytotoxicity is one mechanism by which immunoediting operates within bladder cancers. It is known that many bladder cancers downregulate the expression of MHC class I and II proteins (115, 116), thereby impairing antigen presentation to T cells and immunosurveillance. Thus, cytotoxic T cells may select for less immunogenic clones which have evolved such evasive maneuvers by eliminating more "visible" clones.

We have previously discussed the maturation block of dendritic cells in the context of bladder cancer and given their pivotal role in activating CD8 T cells, this too is a mechanism by which T cell cytotoxicity is restrained. Interestingly, some bladder cancer subtypes have an "immune desert" phenotype and evade immunosurveillance and CD8 mediated cytotoxicity by generating an environment which is hostile to them despite the presence of neo-antigens. A recent genomic analysis in bladder cancer found that activation of the PPARγ, FGFR3 and

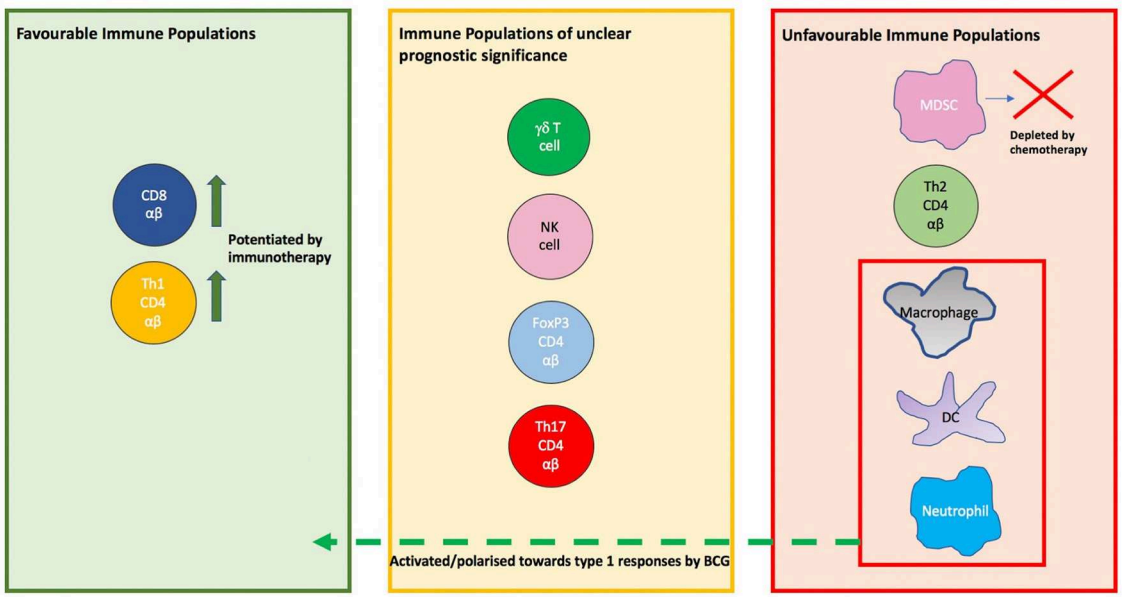

FIGURE 5 | The prognostic significance of immune cell populations in the tumor and modulation by therapies. On the left are populations whose presence in tumor is associated with a favorable outcome such as CD8 cells and Th1 CD4 αβ T cells, with the activity of both potentiated by immunotherapy. On the right are populations whose presence in tumor is associated with tumor promotion. In the red box are populations which are shifted to a anti-tumor phenotype in the context of BCG therapy. In the center box are populations whose prognostic or predictive value in bladder cancer is unclear.

β-catenin pathways in a tumor correlated with poor immune cell infiltration despite a similar somatic mutational load to more heavily infiltrated tumors (8).

As activation of these pathways correlates to the molecular subtype of bladder cancer, this is confirmation of the previously discussed relationship between molecular subtype and immune infiltration. CD8 T cells lie at the end of a long story of activation and polarization of successive immune populations and, given this, any treatments such as checkpoint blockade which target them alone is likely to be limited in efficacy. In addition, their efficacy may be limited by adaptations of the tumor as described above. However, their incredible potential is demonstrated by the small cohort of patients who respond to checkpoint inhibition in urothelial cancer. Future therapeutic strategies would do well to combine multiple approaches along different axes, both innate and adaptive.

CONCLUSION AND FUTURE DIRECTIONS

It is clear from the evidence considered that the immune system is highly active in the context of bladder cancer, however, some of these activities are greatly counter-productive and pro-tumorigenic. The tumor itself is a key immunological player, often profoundly shaping immune responses to favor itself. It is also clear that this is a multi-faceted system with contributions from the lymphoid and myeloid lineages (**Figure 5**). This explains the moderate success of therapies such as checkpoint blockage which target isolated axes within this system. Of note, innate cells of the myeloid lineage are often resident in the bladder and play an important role in shaping the immune response but are oft neglected in research and therapeutics. Future immunotherapeutic strategies have to focus on how the immunosuppressive tumor microenvironment may be curbed, in addition to enhancing the actions of innate immune subpopulations.

Whilst we have considered the extensive evidence for an overall suppression of immunosurveillance responses within the tumor, the understanding of tumor immunoediting *in vivo* is still a nascent study. However, with focus on antigen specific responses in future therapies such as CAR-T cells or peptide vaccines, understanding this process of clonal selection will become ever more important.

Additionally, the bladder is no longer thought to be a sterile organ and there is increasing interest in the urinary microbiome. Though there is some evidence from small studies of differences in the urinary microbiome between patients with bladder cancer and healthy individuals, the evidence is suggestive rather than definitive and no clear mechanisms have been identified (117). With the observation that antibiotic therapy appears to increase your risk of bladder cancer, there will no doubt be more interest in the impact of the urinary microbiome on pathogenesis and treatment for bladder cancer (118).

Perhaps, the most exciting avenues to explore involve combining the treatments which exist, such as BCG or checkpoint inhibition (which are currently being trialed in combination) with additional treatments to modulate the immune response for maximal effect. Thus, it may well be that every little helps in the anti-tumor response.

AUTHOR CONTRIBUTIONS

MJ planned and wrote the manuscript. DE was involved throughout in editing, review, and approval of the manuscript.

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Development of a Prospective Data Registry System for Non-muscle-Invasive Bladder Cancer Patients Incorporated in the Electronic Patient File System

Murat Akand[1,2†], *Tim Muilwijk*[1†], *Jan Cornelissen*[1], *Siska Van Bruwaene*[3], *Kathy Vander Eeckt*[4], *Frederic Baekelandt*[5], *Pieter Mattelaer*[6], *Raf Van Reusel*[7], *Ben Van Cleynenbreugel*[1], *Steven Joniau*[1] *and Frank Van Der Aa*[1*]

[1] Department of Urology, University Hospitals Leuven, Leuven, Belgium, [2] Department of Urology, School of Medicine, Selçuk University, Konya, Turkey, [3] Department of Urology, AZ Groeninge, Kortrijk, Belgium, [4] Department of Urology, AZ Sint Blasius, Dendermonde, Belgium, [5] Department of Urology, AZ Sint Lucas, Bruges, Belgium, [6] Department of Urology, AZ Damiaan Oostende, Oostende, Belgium, [7] Department of Urology, AZ Turnhout, Turnhout, Belgium

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Mieke Van Hemelrijck,
King's College London,
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Sabine Rohrmann,
University of Zurich, Switzerland
Shashwat Sharad,
Center for Prostate Disease Research (CPDR), United States

***Correspondence:**
Frank Van Der Aa
frank.vanderaa@uzleuven.be

[†] These authors have contributed equally to this work and share first authorship

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Purpose: To develop a prospective non-muscle-invasive bladder cancer (NMIBC) data registry by generating NMIBC-specific electronic case report forms (eCRFs) in our institution's electronic patient file system, and to report on the development and implementation of a prospective multicentric registry.

Methods: Templates for data collection, including clinical outcome parameters and quality indicators, were developed in InfoPath™ as an eCRF and were incorporated in our hospital's electronic patient file system. Quality parameters for managing NMIBC patients that were identified by comprehensive literature review were included in the eCRFs. Three separate eCRFs were developed for the management of NMIBC patients: surgery report, bladder instillation form, and multidisciplinary team form.

Results: In August 2013, we started a Flemish prospective clinical and pathological data registry for all patients undergoing transurethral resection of bladder tumor (TURBT) for NMIBC in four participating hospitals, three of which continued using this to date. Three more hospitals started enrolling in 2017, 2018, and 2019, respectively. Written reports of the registered clinical actions are automatically generated within the electronic medical file. When urologists complete these eCRFs, an automated ready-to-send letter to the general practitioner is generated. Up till May 2019, 2,756 TURBTs in 2,419 patients are included in the dataset. Currently, we are recruiting over 600 TURBTs every year.

Conclusions: Easy-to-use eCRFs were developed and included in the electronic patient file system. This registration tool was implemented in 7 hospitals, 6 of which are still using it today. The register harvests important clinical data, while performing routine clinical practice. The data will be used to analyze real-life data of NMIBC patients, to challenge the existing guidelines, to create novel risk stratification tools, and to develop, monitor and validate quality parameters for NMIBC management.

Keywords: bladder cancer, transurethral resection of bladder tumor, database, registry, patient flow

INTRODUCTION

Bladder cancer (BC) is a major health problem, as it is the ninth most commonly diagnosed form of cancer, and accounts for 3% of all cancer-related deaths in Europe (1). The incidence and prevalence increase with age. At first diagnosis, the large majority of detected lesions (±75%) are classified as non–muscle-invasive bladder cancer (NMIBC). These superficial lesions are defined as Ta, T1, or carcinoma *in situ* (CIS). The primary treatment for NMIBC is the removal of all cancerous tissue from the bladder, called transurethral resection of bladder tumor (TURBT), which is used both as a diagnostic and therapeutic tool. Up to 70% of the NMIBC cases will recur, and 15% of all cases will progress in stage and grade (2). Therefore, accurate and early diagnosis of NMIBC is essential to offer the patients the most appropriate treatment and the highest cure rate. For the same reason, NMIBC patients are scheduled to undergo frequent monitoring, currently based on cystoscopy and cytology, which makes BC one of the costliest of all cancers to manage (3, 4).

TURBT is often considered as a straightforward and easy-to-do procedure, and is therefore often treated like a Cinderella (e.g., leaving the procedure to resident). Data suggest that there is wide variability in the quality of TURBTs performed in different centers (5). Several recommendations have been made for modifying the TURBT technique with the ultimate aim to increase its quality (6–8). Although three TURBT checklists have been proposed to improve the quality of the operation, only the 10-item one developed by Anderson et al. has been evaluated in clinical practice (9–12).

Current guidelines are based on relatively small prospective patient cohorts with medium-term follow-up. The European Organization for Research and Treatment of Cancer (EORTC) risk calculator, which predicts the short- and long-term risks of disease recurrence and progression, is the result of a *post-hoc* statistical analysis of 2,596 patients, treated between 1979 and 1989, from seven separate prospective trials with 291 to 517 included patients (13). They were categorized by the old (pre-2004) WHO grading system. Because only a minority ($n = 171$) patients in the EORTC cohort were treated with Bacillus Calmette–Guérin (BCG) and none of them received maintenance treatment (which is now considered mandatory for at least 12 months to lead to an effect), the Spanish CUETO consortium (Club Urologico Español de Tratamiento Oncologico) developed another risk stratification model that predicts the risk of recurrence and progression based on a total of 1,062 patients treated with BCG between February 1990 and May 1999 in 4 prospective trials (14). Both risk calculators tend to overestimate the risk of disease recurrence and progression in high-risk patients and have poor discrimination for prognostic outcomes in external validation (15, 16).

Based on the known risk factors, NMIBC patients are stratified into three risk categories: low-, intermediate- and high-risk. Treatment recommendations are guided by this stratification (17). Management of intermediate- and high-risk NMIBC consists of TURBT and bladder instillations with BCG plus intensive follow-up and maintenance BCG. Despite this intensive treatment and follow-up schedule, these patients have a high risk for disease recurrence and a moderate to high risk for progression to muscle-invasive bladder cancer (MIBC) of up to 35–55% at 5-year follow-up (17).

The care of NMIBC patients is complex. Even in modern medicine, concerns have been raised regarding the variation in management of patients with BC (18). Population based data have shown the real-life survival is lower than expected from clinical trials (19, 20). A clear patient flow chart with predefined outcome parameters and quality indicators is expected to improve overall patient care. The current major challenge and unmet need is prospective real-life collection of NMIBC patient data. There is need for robust reporting rules and robust internally and externally validated prediction models based on up-to-date datasets. As timely updating of the currently used and above-mentioned datasets is impossible (as they are *post-hoc* analyses of terminated trials), a prospective dataset needs to be developed.

Keeping these unmet needs and deficiencies of the former risk stratifications in mind, we developed a prospective NMIBC data registry by generating NMIBC-specific electronic case report forms (eCRFs). With this registry, we aimed to benchmark the current standard of care with existing guidelines, and also collect high-quality data to develop a novel prediction model. In this manuscript, we report on the development of these eCRFs and their implementation in a prospective multicentric registry.

MATERIALS AND METHODS

Electronic forms for data collection, including relevant clinical outcome parameters and quality indicators, were developed in InfoPath™ (Microsoft Corporation, Redmond USA). InfoPath forms serve as interface in front of the electronic patient file system. These forms are used as an eCRF. To comply with local privacy laws, all data is stored in the hospital's electronic patient file system itself (called Klinisch Werkstation (KWS), which runs in different Flemish hospitals), which is protected by firewalls. The eCRFs have been developed based on the recommendations of the European Association of Urology (EAU) guidelines and the Canadian Urological Association (CUA) white paper by consensus of two academic and one non-academic urologists (17, 18).

Three separate eCRFs were developed for the management of NMIBC patients: a surgery report form, bladder instillation form, and multidisciplinary team (MDT) form. The data collected in these forms are listed in **Tables 1–3**, respectively. Besides scientific outcome parameters and quality indicators, specific attention was paid to patient comorbidities by systematically including Charlson Comorbidity Index and smoking status. These eCRFs serve for daily clinical practice and for prospective data registration at the same time. With automatically generated ready-to-send letters, they provide standardized data collection while not increasing the workload of the urologist. Data is extracted trough algorithms with pseudonymization for centers and anonymized for intercenter sharing. This registry has been registered in ClinicalTrials.gov (NCT03973671).

TABLE 1 | The data collected in the operation form.

- Patient demographics (Name, Age, Sex, ID number) (automatically filled in)
- Date (automatically filled in)
- Name of supervisor*
- Name of assistant*
- Operation type*

❖ URS
- ○ Diagnostic
  - ▪ Lesion on right side*
  - ▪ Lesion on left side*
  - ▪ Localization and number (for each side)*
    - ◊ A table of 6 lines for localization (lower ½ ureter, upper ½ ureter, renal pelvis, lower pole, middle pole, upper pole) and 5 columns of number (0,1,2,3,≥4), and 1 column for macroscopic invasive appearance
    - ◊ Number of total tumors (automatically calculated)
  - ▪ Selective cytology
  - ▪ Biopsy
  - ▪ Dimension of the largest lesion
  - ▪ *Additional notes*
- ○ Therapeutic
  - ▪ Lasering on right side
    - ◊ Complete
    - ◊ Incomplete
  - ▪ Lasering on left side
    - ◊ Complete
    - ◊ Incomplete
- ○ Conclusion and further planning
  - ▪ Imaging of the upper urinary tract
    - ◊ CT→ with/without cytology
    - ◊ MR→ with/without cytology
  - ▪ Re-URS (→ …. *weeks later*)
  - ▪ Nefro-ureterectomy
  - ▪ Wait and see
- ○ New action application→ MDT (date)

❖ TURBT
- ○ *Operation duration (min)*
- ○ Operator
- ○ Cytology result*
  - ▪ Positive
  - ▪ Negative
  - ▪ Not representative
  - ▪ Not taken
  - ▪ Not known
- ○ Use of Hexvix*
  - ▪ No
  - ▪ Yes
    - ◊ Hexvix avide lesions (No, Yes)
    - ◊ Extra visualized lesion (No, Yes→ *Number*)
- ○ Examination under anesthesia performed*
  - ▪ No
  - ▪ Yes
    - ◊ Normal
    - ◊ Divergent (→ *Brief description*)

(Continued)

TABLE 1 | Continued

- ○ Type of anesthesia
  - ▪ Spinal
  - ▪ General
- ○ Intervention type*
  - ▪ First TURBT (±ad random biopsies)
  - ▪ TURBT for residual disease
    - ◊ In the last year
    - ◊ More than 1 year ago
  - ▪ Re-TURBT (Intermediate/high risk)
    - ◊ Ad random biopsies
    - ◊ TUR for scar tissue
    - ◊ New TCC lesion
    - ◊ Incomplete resection (at previous TURBT)
    - ◊ No detrusor muscle at previous resection
- ○ TCC localization*
  - ▪ Suspicious TCC lesion
  - ▪ Atypical lesion (*Brief description*)
  - ▪ Localization and number
    - ◊ A table of 8 lines for localization (anterior, posterior, right, left bladder wall, dome, base, bladder neck, prostatic loge) and 6 columns of number (0,1,2,3,4,≥5), 1 column for diffuse spreading TCC lesion, and 1 column for suspected CIS/red zone
    - ◊ Number of total tumors (automatically calculated)
  - ▪ Dimension of the largest lesion
    - ◊ <1 cm
    - ◊ 1–3 cm
    - ◊ >3 cm
- ○ Resection of ostium
  - ▪ No
  - ▪ Yes
    - ◊ Left→ DJ stent placement (No / Yes)
    - ◊ Right→ DJ stent placement (No / Yes)
- ○ Conclusion and Further planning
  - ▪ Specimen sent to pathology*
    - ◊ No
    - ◊ Yes
  - ▪ Macroscopically complete resection*
    - ◊ No
    - ◊ Yes
  - ▪ Macroscopically muscle invasion*
    - ◊ No
      - ✓ Ta
      - ✓ T1
    - ◊ Yes (T2)
  - ▪ Presumed differentiation grade*
    - ◊ G1 ◊ PUNLMP
    - ◊ G2 ◊ LG
    - ◊ G3 ◊ HG
  - ▪ Complication
    - ◊ Bleeding
    - ◊ Other (Brief description)
  - ▪ Bladder perforation*
    - ◊ No

(Continued)

TABLE 1 | Continued

- ◊ Yes
- Transurethral catheter with continuous irrigation*
  - ◊ No
  - ◊ Yes
- Provisionary EORTC recurrence score (automatically calculated)
- Postoperative single instillation of Mitomycin C*
  - ◊ Yes
  - ◊ No→ Reason
    - ✓ Continuation of bladder irrigation
    - ✓ Perforation
    - ✓ Incomplete resection
    - ✓ Very deep/extensive resection
    - ✓ Presumption of no bladder TCC
    - ✓ High-risk TCC/already received BCG
    - ✓ Muscle-invasive TCC
    - ✓ Known BCG intolerance
    - ✓ Patient comorbidity
    - ✓ Functional bladder problem
    - ✓ Surgeon's choice
    - ✓ Other (→*Brief description*)
- *Additional notes*
- New action application→MDT (date)

❖ Others

- DJ insertion*
- Urethra dilatation*
- Other (→ *Brief description*)
- New action application→ MDT (date)

Underlined parameters are chosen from the drop-down menus. Parameters in italics need to be written manually. Automatically filled in and automatically calculated parameters are mentioned in parenthesis, and are colored in black and red, respectively. Answers for all other parameters are clicked from the options listed below the parameters. Items marked with an asterisk (*) are mandatory fields.

Biannual consensus meetings with members of the network (Vlaams Ziekenhuis Netwerk KU Leuven, VZNKul) were carried out to consent on reporting forms and discuss registry related topics. As such, a complete digital patient flow registration with scientific output parameters and with quality indicators based on the current knowledge has been developed. Patient flow-charts for the diagnosis of bladder cancer, and management of low-, intermediate- and high-risk NMIBCs are shown in **Supplementary Figures 1–4**.

Quality parameters for NMIBC management were identified through a comprehensive review of literature. For the review of quality parameters, a literature search including case control studies, cohort studies, randomized controlled trials (RCTs), systematic reviews and meta-analyses, was conducted on PubMed/Medline and Embase databases in March 2013. This comprehensive review has been renewed in March 2018, and was recently published (21). Currently selected quality indicators are listed in **Table 4** (21, 22).

This registry was developed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The confidentiality of patient data

TABLE 2 | The data collected in the instillation form.

- Patient demographics (Name, Age, Sex, ID number) (automatically filled in)
- Date (automatically filled in)
- Name of supervisor
- Name of assistant
- Name of nurse
- Instillation type*
  - Postoperative single instillation of MMC
  - Induction schema for chemotherapy
  - Maintenance treatment for chemotherapy
  - Induction schema for BCG
  - Maintenance treatment for BCG
  - Interstitial cystitis/Bladder pain syndrome/Radiocystitis
- Catheter type and dimension
- Session number*
- Instillation product and dosage*
  - Chemotherapy
    - Mitomycin C (40 mg, 1/2, 1/3)
    - Epirubicine (50 mg, 80 mg, 1/2)
    - Doxorubicine (50 mg, 1/2, 1/3)
    - Gemcitabine (2 g, 1/2, 1/3)
  - BCG
    - OncoTice (12.5 mg, 1/2, 1/3)
    - BCG Medac (50 mL, 1/2, 1/3)
    - Immunocyst (81 mg, 1/2, 1/3)
- Instillation not administered
  - Reason*
    - Suspicion of UTI
    - Hematuria
    - Bladder perforation
    - According to doctor's advice
    - MMC not delivered
    - Patient did not come
    - Patient's intolerance
  - Date of the new instillation
- Residual urine beforehand
  - No
  - Yes→ *Volume (ml)*
- Patient's complaints*
  - No
  - Yes
    - Macroscopic hematuria
    - UTI
    - LUTS (without a sign of infection)
    - Fever
    - Dyspnea
    - Arthralgia
    - Other (*Brief description*)
- Dipstick test performed
  - No
  - Yes
    - Leucocyte esterase
      - ◊ Positive

(Continued)

TABLE 2 | Continued

- ◊ Negative
- Nitrite
  - ◊ Positive
  - ◊ Negative

- Post-instillation
  - How did the instillation go*
    - Smooth
    - Troublesome but atraumatic catheterization
    - Traumatic catheterization
  - Experience of pain (from 1 to 10)
  - Urine culture taken/performed
    - Yes
    - No
  - Presence of residue afterwards
    - Yes
    - No
  - Planned next instillation
    - Yes→ Date
    - No→ Reason
      - ◊ End of schema
      - ◊ Intolerance
      - ◊ Traumatic catheterization
      - ◊ Due to contraindication

Underlined parameters are chosen from the drop-down menus. Parameters in italics need to be written manually. Automatically filled in and automatically calculated parameters are mentioned in parenthesis, and are colored in black and red, respectively. Answers for all other parameters are clicked from the options listed below the parameters. Items marked with an asterisk (*) are mandatory fields.

was guaranteed. The registry protocol was first approved by the institutional review board (Clinical Trials Center [CTC] UZ Leuven) in August 2013. With amendments, the registry was finally approved by the Ethics Committee Research UZ/KU Leuven (approval date: 06/06/2014, approval number: S55725). According to the General Data Protection Regulation (GDPR), written informed consent is obtained from every included patient.

RESULTS

The first version of the registry was generated by using the standardized surgery report forms in August 2013 for all patients undergoing TURBT for NMIBC in four participating hospitals. Patient flow-charts for different risk categories were written and visualized on the intranet. With the addition of eCRF for bladder instillation in April 2016 and eCRF for MDT in September 2016, we started to use the second version of the registry system. The fifth to seventh hospital started enrolling patients in Q2 2017, Q1 2018, and Q1 2019, respectively. Several other hospitals are in the process of starting up the KWS system as a hospital-wide electronic patient file system and will start to enroll patients as soon as that process is completed. One of the four initial hospitals stopped recruiting patients after fusion with another

TABLE 3 | The data collected in the MDT form.

GENERAL

- Patient demographics (Name, Age, Sex, ID number) (automatically filled in)
- *Patient height and weight*→ BMI (automatically calculated)
- Date of contact/MDT
- Previous procedure*
  - No
  - Yes
    - Date
    - First TURBT
      - ◊ Ad random biopsies
    - TURBT for residual disease
      - ◊ In the last year
      - ◊ More than 1 year ago
    - Re-TURBT
      - ◊ Ad random biopsies
      - ◊ Scar tissue
      - ◊ New TCC lesion
      - ◊ Incomplete resection (at previous TURBT)
      - ◊ No detrusor muscle at previous resection
- Type of MDT*
  - First
    - With surgery
    - Without surgery
  - Repeating- - - - - - - - - - - - >If this option is chosen, data about the risk category, stage, grade, presence of CIS, number of lesions, prior recurrence rate from the previous MDT form is shown automatically.
    - With surgery
      - ◊ Residual
      - ◊ Re-TUR (high risk)
    - Without surgery
- Second opinion
- Charlson comorbidity index calculator* (the score, estimated relative risk of death, and probability of survival after 1 and 2 years are automatically calculated)
- *Medical history*
- *Current medication*
- Kidney function (automatically retrieved from the laboratory module during the first creation of the form)
  - Last creatinine level (with date)
  - Last eGFR level (with date)
- Smoking status*
  - Active→ *Pack year*
  - Ex→ *Pack year*
  - Never
  - Not known
- *Additional notes*

CURRENT LESION

- Cytology
  - Malignant
  - Non-malignant
  - Not known
  - Not performed
- Result of recent upper urinary tract imaging
  - No

(Continued)

TABLE 3 | Continued

- ○ Yes
  - . CT/IVU→ Date
  - . MR/IVU→ Date
  - . Bladder lesion
    - ◊ No
    - ◊ Yes
  - . Lymph nodes
    - ◊ No
    - ◊ Yes
  - . Upper urinary tract
    - ◊ TCC lesion
      - ✓ No
      - ✓ Yes→ Right / Left
    - ◊ Hydronephrosis
      - ✓ No
      - ✓ Yes→ Right / Left
- Risk determination
  - ○ Date of last intervention
  - ○ Stage*
    - . T0
    - . Ta
    - . T1
    - . ≥T2
  - ○ Grade*
    - . G1 . PUNLMP
    - . G2 . LG
    - . G3 . HG
  - ○ Carcinoma *in situ**
    - . Absent
    - . Present
  - ○ Lymphovascular invasion
    - . No
    - . Yes
  - ○ Micropapillary variant
    - . No
    - . Yes
  - ○ Detrusor muscle present in resection*
    - . No
    - . Yes
  - ○ Complete resection*
    - . No
    - . Yes
  - ○ Diffuse lesion
    - . No
    - . Yes
  - ○ Dimension of the largest lesion*
    - . <1 cm
    - . 1–3 cm
    - . >3 cm
  - ○ *Number of lesions**
  - ○ Adjuvant MMC given*
    - . No
    - . Yes

(Continued)

TABLE 3 | Continued

- ○ Residual disease*
  - . No
  - . Yes
- ○ Risk of current lesion → Low risk, Intermediate risk, High risk
- ○ Category that will determine advice → Low risk, Intermediate risk, High risk

MDT ADVICE

- Advice of the MDT→ Low risk, Intermediate risk, High risk (According to disease risk, one of three different forms is opened)

❖ LOW RISK

- Cystoscopy date*
- Deviation from standard protocol*
  - ○ No
  - ○ Yes
    - . UUT imaging
    - . Cytology
    - . Adjuvant instillation

❖ INTERMEDIATE RISK

- UUT screening*
  - ○ No
  - ○ Immediately
  - ○ Yearly
- Adjuvant instillation*
  - ○ No
  - ○ Yes
    - . Type
      - ◊ MMC
      - ◊ BCG
    - . Schema
      - ◊ 1 year
      - ◊ 3 years
    - . Dosage
    - . Start date

❖ HIGH RISK

- Re-TURBT*
  - ○ No → Reason*
    - . Small lesion
    - . Previous resection
    - . Patient comorbidity
    - . Early cystectomy
    - . CIS
    - . No malignancy
    - . Focal high grade
    - . Other
      - → Adjuvant BCG instillation planned* (automatically opened as "No" is clicked for Re-TURBT)
    - . No
      - ◊ Reason
        - ✓ Known intolerance
        - ✓ Patient comorbidity
      - ◊ Other therapy
        - ✓ MMC instillation
        - ✓ Others (hyperthermia/…) (→ *Brief description*)

(Continued)

TABLE 3 | Continued

- ✓ None
  - Yes
    - ◊ Induction schema
    - ◊ Maintenance schema
  - Yes→ Date
    - ± re-TUR of scar
    - ± ad random biopsy
    - ± biopsy of prostate loge
- Yearly UUT screening*
  - No→ Warning pop-up screen: "Deviation from standard protocol!"
    Reason
  - Yes
    - CT
    - MRI
- *Additional notes*

Underlined parameters are chosen from the drop-down menus. Parameters in italics need to be written manually. Automatically filled in and automatically calculated parameters are mentioned in parenthesis, and are colored in black and red, respectively. Answers for all other parameters are clicked from the options listed below the parameters. Items marked with an asterisk (*) are mandatory fields.

TABLE 4 | Selected quality indicators.

- Time between diagnosis of NMIBC and TURBT (percentage of patients that received surgery within 3 weeks)
- Percentage of patients that underwent complete resection
- Percentage of patients for whom the surgical report documents on visual completeness of the TURBT, depth of TURBT and examination under anesthesia findings
- Percentage of patients that undergoes adjuvant Mitomycin C instillation after complete TURBT
- Timing between surgery and instillation of adjuvant Mitomycin C (percentage of patients within 6 h and within 24 h)
- Percentage of pathology reports available within 1 week of TURBT
- Percentage of pathology reports noting detrusor muscle in pathologic specimen (for high-risk tumors)
- Percentage of newly diagnosed intermediate- and high-risk NMIBC patients that underwent upper tract imaging within 1 month before or after TURBT
- Percentage of patients with high-risk NMIBC and whose pathology report noted absence of detrusor muscle that underwent restaging TURBT within 6 weeks of initial resection
- Percentage of early recurrences
- Percentage of patients that started BCG, percentage of patients that completed 12 months of maintenance BCG
- Time between decision for early cystectomy and cystectomy

non-participating center that is currently not using the same electronic patient file system.

The treating urologists make an update of the database while performing routine clinical practice. Written reports of the registered clinical actions are automatically generated and are incorporated in the medical file. When urologists complete these eCRFs, an automated ready-to-send letter to the general practitioner is generated. This automated letter motivates participating urologists to complete these eCRFs and ensures correct and complete data collection for all patients while providing an important time gain for the urologists. In all three forms, essential fields are mandatory to fill in, which ensures all relevant data to be collected properly. Automated pop-up windows warn the physician when a deviation from the standard-of-care management flow occurs.

Up till May 2019, 2756 TURBTs have been registered in 2,419 patients. The number of all TURBTs registered by each center according to registry version is listed in **Supplementary Table 1**. The numbers of all registered TURBTs and unique patients per each year are listed in **Supplementary Table 2**. The numbers of all registered bladder instillations and unique patients per year are listed in **Supplementary Table 3**.

The goal of the program is to continue the registry in order to have a large number of included patients with long follow-up. The power of the registry increases with time and with addition of other network hospitals using KWS database. Currently, we expect to collect clinical, pathological and outcome data for around 600 patients per year using eCRFs for TURBTs, bladder instillations and MDTs.

DISCUSSION

Collection of real-life data from cancer patients is a critical step of patient management and clinical science. Reliability due to accurately and timely collection of data, easiness to use the system and to evaluate the harvested data, and security of the stored data define the robustness of such a patient database. In the past, registration of patient data used to be a manual task. The number of qualitative registries is increasing with the implementation of electronic patient file systems, which allow easier and faster capture of data. Well-designed registries are a good way to collect and to analyze cancer survivorship in a real-life setting, and they have an added value next to randomized controlled studies (23–27). The population-based registries may either cover a region (California Cancer Registry), a country (SEER [Surveillance, Epidemiology, and End Results], NSQIP [The American College of Surgeons National Surgical Quality Improvement Program], SNRUBC [Swedish National Register of Urinary Bladder Cancer]) or a group of countries (EUROCARE-5).

EORTC and CUETO risk calculators tend to overestimate the risk of disease recurrence and progression in high-risk patients and have poor discrimination for prognostic outcomes in external validation. Recurrence and progression rates from current patients differ from those calculated from historical patient cohorts (15, 16), and therefore, need to be re-determined on patient cohorts that are categorized and treated according to the current state of the art. Based on the new data generated in this registry, we will try to address this by developing a new risk calculator, which will be readily available for Flemish hospitals to use.

NMIBC patients are often not treated according to the guidelines, because the management pathway given in these guidelines is complex, and establishment of a good patient flow is logistically difficult. We hypothesize that by standardizing the

patient flow, especially with surgery report and MDT report, and monitoring it, these deviations from the guidelines-based follow-up will be structured and we can learn where and why these guidelines are not followed. Moreover, by rolling out the registry in different hospitals, we will find practice variation and be able to analyze (and eventually remediate) it.

The registry can be of direct value for the treatment of NMIBC patients in Flanders and on the long run even worldwide. We expect the eCRFs to have effect on three different but interrelated levels of the management of NMIBC:

1) Daily clinical practice: These eCRFs will ensure the urologists to make complete and standardized reporting of their patients, while decreasing their workload with easy-to-use style and automatically generated ready-to-send letters. Moreover, it will help to diminish deviations from standardized care paths. At the same time, each individual patient will benefit from this complete, standardized reporting and better risk stratification by having guidelines-based, state-of-the-art treatment. The registry will also provide high quality long-term follow-up data of the patients.
2) Centers: This registry will allow the participating centers to check their quality control of patient flow in NMIBC diagnosis and treatment, to monitor their adherence to the EAU guidelines, to detect internal practice variation, and to benchmark themselves with the other urology departments in regards of several outcome parameters and quality control parameters.
3) Knowledge of the disease: With the queries generated within the continuously growing real-life dataset, it will be able to reflect the current practice, to monitor guideline deviations and analyze them, to re-determine recurrence and progression rates, to serve as validation data for other calculators, and even to develop a novel risk calculator.

As long as TURBTs, MDTs and instillations are performed in the participating centers, the dataset will be continuously updated and enlarged. The treating urologist updates the database by merely writing the TURBT report, instillation report and MDT report. There is no extra action required. The number of included patients and follow-up will rapidly and highly exceed the current datasets used in the field. The EORTC and CUETO datasets have a median follow-up of 3.9 years and 69 months, respectively. From the initial start of the surgery registration as a pilot study (August 2013) up till May 2019, 2,756 TURBTs on 2,419 patients have already been included in the dataset. The long-term follow-up will allow more robust recurrence, progression and even survival data. Moreover, this registry can immediately be expanded to other centers that are using KWS in Flanders and to other (inter)national centers that are willing to use the same reporting standards. As such, the number of included patients per year is expected to increase in the years to come since more and more hospitals are joining healthcare networks.

The eCRFs include nine of the ten mandatory items (excluding tumor characteristics such as sessile, nodular, papillary, flat) and two of the three optional items (excluding separate deep biopsy sent from resection bed) of the checklist developed by Anderson et al. (11). The important features that make the registry unique are: (i) collection of all relevant clinical, pathological and follow-up data of the NMIBC patients, (ii) being completely implemented in the electronic patient file system, (iii) user friendly style with drop-down menus and clicking boxes used for the vast majority of the parameters and very few parameters to be entered by writing, iv) not missing data with all essential fields being mandatory, (v) warning the urologist with pop-up windows when a deviation from the standard of care occurs, and (vi) preparing a ready-to-send letter to the general practitioner that decreases the workload of the urologist. We think that this registry will ensure a better management of NMIBC patients while allowing us to collect robust and reliable data that can be used in various trials. Moreover, we are now building a pathology report to be filled in by pathologists for more detailed pathological reporting.

On the other hand, this registry is not devoid of limitations. First, it is readily available in KWS system, which may limit its use. However, it can be implemented into other electronic patient file systems with appropriate IT support. Second, automated queries can be performed for most of the quality parameters (21, 22), while a few ones have to be queried manually. This deficit can be compensated by an improvement in the software. And last, it currently includes only NMIBC patients, however, we are in the process of developing eCRFs for MIBC patients.

CONCLUSION

The easy-to-use eCRFs, which generate automated letters to general practitioners, harvest important data that will be used to define real-life data of NMIBC patients, to challenge the existing guidelines, to create a novel risk stratification tool, and to monitor the quality parameters for NMIBC patient flow. We hope that this registry can be disseminated to more urology departments in the near future, and also sets a precedent to further registries in different departments/diseases.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This registry was developed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The confidentiality of patient data was guaranteed. The registry protocol was first approved by the institutional review board (Clinical Trials Center [CTC] UZ Leuven) in August 2013. With amendments, the registry was finally approved by the Ethics Committee Research UZ/KU Leuven (approval date: 06/06/2014, approval number: S55725). According to the General Data Protection Regulation (GDPR), written informed consent is obtained from every included patient.

AUTHOR CONTRIBUTIONS

FV, MA, TM, JC, SV, and KV contributed to the conception and design of the study. FV, JC, SV, KV, FB, PM, RV, and BV organized the database. MA and TM wrote the first draft of the manuscript. FV and SJ edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01402/full#supplementary-material

Supplementary Figure 1 | Patient flow-chart for the diagnosis of bladder cancer and treatment with TURBT. NMIBC, Non-muscle-invasive bladder cancer; eGFR, Estimated glomerular filtration rate; CT IVU, Computed tomography – intravenous urography; UUT TTC, Upper urinary tract transitional cell carcinoma; TURBT, Transurethral resection of bladder tumor; MRI, Magnetic resonance imaging; MMC, Mitomycin C; MDT, Multidisciplinary team; CIS, Carcinoma in situ; LG, Low grade; MIBC, Muscle-invasive bladder cancer.

Supplementary Figure 2 | Patient flow-chart for the management low-risk of bladder cancer. MDT, Multidisciplinary team; TURBT, Transurethral resection of bladder tumor.

Supplementary Figure 3 | Patient flow-chart for the management intermediate-risk of bladder cancer. MDT, Multidisciplinary team; TURBT, Transurethral resection of bladder tumor; UUT, Upper urinary tract; CT IVU, Computed tomography – intravenous urography; MMC, Mitomycin C; BCG, Bacillus Calmette-Guérin.

Supplementary Figure 4 | Patient flow-chart for the management high-risk of bladder cancer. MIBC, Muscle-invasive bladder cancer; CIS, Carcinoma in situ; BCG, Bacillus Calmette-Guérin; TURBT, Transurethral resection of bladder tumor; ADR, Ad random biopsy; MDT, Multidisciplinary team; UUT, Upper urinary tract.

Supplementary Table 1 | The distribution of the TURBTs registered by each center according to registry version (till the beginning of May 2019).

Supplementary Table 2 | The number of all TURBTs and unique patients per year.

Supplementary Table 3 | The number of all bladder instillations and unique patients per year.

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SYSTEMATIC REVIEW
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Clinical Significance of Pre-treated Neutrophil-Lymphocyte Ratio in the Management of Urothelial Carcinoma: A Systemic Review and Meta-Analysis

*Jungyo Suh[1,2], Jae Hyun Jung[1], Chang Wook Jeong[1], Cheol Kwak[1], Hyeon Hoe Kim[1] and Ja Hyeon Ku[1]**

[1] Department of Urology, Seoul National University College of Medicine, Seoul, South Korea, [2] Department of Urology, Seoul Metropolitan Government- Seoul National University Boramae Medical Center, Seoul, South Korea

Purpose: We performed a study-level meta-analysis to summarize the current evidence on the correlation between pretreatment neutrophil-to-lymphocyte ratios (NLR) and oncological outcomes in each type of management for urothelial carcinoma.

Method: All articles published until February 2017 in PubMed, Scopus, and EMBASE database were collected and reviewed. The current evidence on correlations between pretreatment NLR and oncological outcomes in each type of management for urothelial carcinoma, including transurethral resection of bladder tumor (TURBT), radical cystectomy (RCx), chemotherapy (CTx), and nephroureterectomy (NUx), were summarized.

Results: Thirty-eight studies containing clinical information on 16,379 patients were analyzed in this study. Pooled hazard ratios (HR) and odds ratios (OR) with 95% confidence intervals were calculated after weighing each study. Heterogeneity among the studies and publication bias were assessed. Pretreatment NLR was significantly associated with muscle invasiveness (OR: 4.27), recurrence free survival (RFS, HR: 2.32), and progression-free survival (PFS, HR: 2.45) in TURBT patients. In the RCx patients, high NLR was negatively associated with both disease status (extravesical extension and lymph-node positivity, OR: 1.14 and 1.43, respectively) and oncological outcomes [overall survival (OS), PFS], and cancer specific survival (CSS, HR: 1.18, 1.12, and 1.35, respectively). Pretreatment NLR was negatively correlated with pathologic downstaging (OR: 0.79) and positively correlated with PFS (HR: 1.30) and OS (HR: 1.44) in CTx patients. For patients who underwent NUx, pretreatment NLR was significantly associated with OS (HR: 1.72), PFS (HR: 1.63), and CSS (HR: 1.68).

Conclusions: Pretreatment NLR is a useful biomarker for disease aggressiveness, oncological outcome, and treatment response in the management of patients with urothelial carcinoma. More evidence is needed to clarify these results.

Keywords: urothelial carcinoma, neutrophil-lymphocyte ratio, trans-urethral resection of bladder tumor, cystectomy, chemotherapy, oncological outcome

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***Correspondence:**
Ja Hyeon Ku
randyku@hanmail.net

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INTRODUCTION

Urothelial carcinoma of the bladder is the third most common and the eighth most lethal malignancy in the United States (1), showing a high incidence in developed countries (2). This malignancy originates from normal urothelial cells and can occur in any part of the urinary tract, including the renal pelvis, ureter, bladder, and urethra. The majority of urothelial carcinomas is present in the lower urinary tract, mostly in the bladder. Muscle invasiveness is the most important parameter in the management of bladder urothelial carcinoma. Non-muscle invasive bladder cancer (NMIBC) can be managed by transurethral resection of bladder tumors (TURBT). Muscle-invasive bladder cancer (MIBC) is treated with radical surgery or systemic treatment. The standard treatment for upper urinary tract urothelial carcinoma (UTUC) is radical nephroureterectomy with bladder cuffing. Since urothelial carcinoma shows a variety of clinical manifestations, selecting the proper treatment is one of the most critical issues in the management of patients with urothelial carcinoma.

Recent studies have revealed that the inflammatory response plays an essential role in tumor development, progression, and prognosis (3). Elevation of C-reactive protein (CRP), the presence of some cytokines, and changes in the proportion of white blood cells in the peripheral blood are common findings reflecting the systemic inflammatory response. Among these changes, the peripheral neutrophil-to-lymphocyte ratio (NLR) is one of the most widely studied prognostic biomarkers in many solid tumors because of its easy calculation and cost-effectiveness (4). High NLR tends to be negatively correlated with poor survival in urothelial carcinoma (5). Some studies have suggested a link between high NLR with the pathologic stage of urothelial carcinoma, including muscle invasiveness (6, 7), extravesical extension (8–10), and lymph-node positivity (10, 11). Moreover, recent research has indicated that NLR is a predictive biomarker of treatment response (12). However, one large-scale study did not find a correlation between high preoperative NLRs and extra-vesical extension (11). Thus, the clinical utility of preoperative NLR as a prognostic or predictive biomarker in urothelial carcinoma is still controversial.

Therefore, the objective of this study was to determine the correlation between pretreatment NLRs and oncological outcomes in each type of management for urothelial carcinoma, including TURBT, radical cystectomy (RCx), chemotherapy (CTx), and nephroureterectomy (NUx), through a meta-analysis of published studies.

MATERIALS AND METHODS

Search Strategy

This study was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The PubMed, SCOPUS, and EMBASE databases were searched to collect suitable literature published up to February 2017. The search used a combination of "Neutrophil; Lymphocyte; Bladder; Cancer" and "Neutrophil; Lymphocyte; Urothelial; and Cancer" terms. To identify additional shrouded studies, we carefully examined the references of each article.

Inclusion and Exclusion Criteria

Two reviewers (CWJ and CK) screened all titles and abstracts of the initially searched articles. After screening, the full-text articles were separately evaluated by two different reviewers (HHK and JHK) to determine study eligibility. Disagreement on study eligibility was resolved through discussion. The inclusion criteria for eligibility were that the report: (1) investigated urothelial carcinoma, (2) had patient neutrophil-to-lymphocyte ratios, (3) evaluated the relationship between pathologic features and prognosis, and (4) had enough information to calculate odds ratios (OR), or hazard ratios (HR) with 95% confidence intervals (CI). Studies not written in English, case reports, editorial letters or reviews, and those not performed in humans were excluded. If the investigations were conducted on similar patients by the same research group, only the largest and newest article was included in the systematic review.

Data Extraction and Handling

Two reviewers (JHJ and JS) independently extracted information from the selected studies. Data tables were constructed to record all associated data from the texts, tables, and figures of each study. The study information (name of authors, publication year, region), the number of patients, follow-up information, and disease status were obtained. After completing the data tables for each study, both reviewers compared their results and arrived at a consensus for any differences.

Statistical Analysis

The DerSimonian and Laird random-effects model (13) was selected to weigh each study for the meta-analysis. Odds ratios and 95% CIs were used to assess the relationship between NLR and disease status for each specific situation, including muscle invasion in TUR, extravesical extension, or lymph node positivity in radical cystectomy, and pathologic downstaging after neoadjuvant chemotherapy. Hazard ratios and 95% CI were used to estimate the effects of NLR on survival. The heterogeneity of the studies was assessed by Chi-squared tests. Statistical significance was considered for p-values of <0.05. The I^2 statistic was also calculated to determine the heterogeneity of the studies. I^2 values larger than 75%, <25%, and between 25 and 75% indicated a high risk of heterogeneity among studies, no heterogeneity, and moderate heterogeneity among studies, respectively.

Publication bias was assessed by funnel plots, rank correlation analysis (Begg test), and linear regression analysis (Egger test). In the funnel plots, a symmetric, inverted funnel shape indicated no publication bias. A p-value < 0.05 by Begg and Egger's test was considered publication bias (**Supplementary Data 1**). The pooled ORs and HRs of the meta-analyses were calculated using RevMan 5.0 (the Cochrane Collaboration, Copenhagen) software. All statistical analyses were performed with R program 3.5.0 (R Development Core Team, Vienna, http://www.R-project.org).

Data quality and the risk of bias assessment were performed by three investigators (JHK, JS, and JHJ). Each reviewer independently read the published articles and performed a quality assessment based the Newcastle-Ottawa Scale (NOS) (14). The NOS assesses the methodologic quality of each study in three domains: selection of the study groups, comparability of the groups, and ascertainment of exposure and outcome. The risk of bias was stratified on three levels. Quality scores of NOS of >7, 4 to 6, and< 4 indicated high quality, moderate quality, and low-quality studies, respectively. The methodologic quality scores of all included studies are shown in **Figure 1**. Specific quality assessment scores and data are shown in **Supplementary Datas 2**, **3**.

RESULTS

Literature Search and Study Selection

A total of 675 articles was primarily identified by database searching. After removing duplicated work, 343 articles remained for screening. After reviewing the running title and abstract by

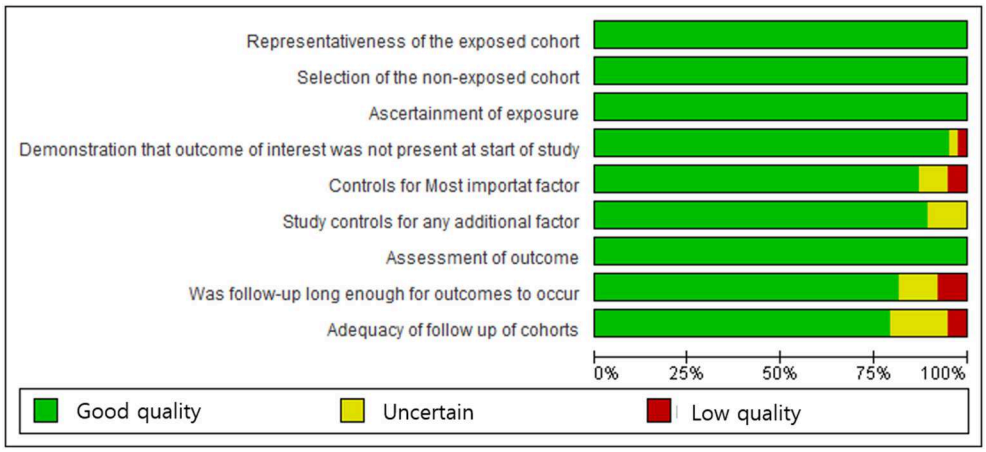

FIGURE 1 | Newcastle-Ottawa Scale graph: the review authors' judgments on each parameter are presented as percentages across all included studies.

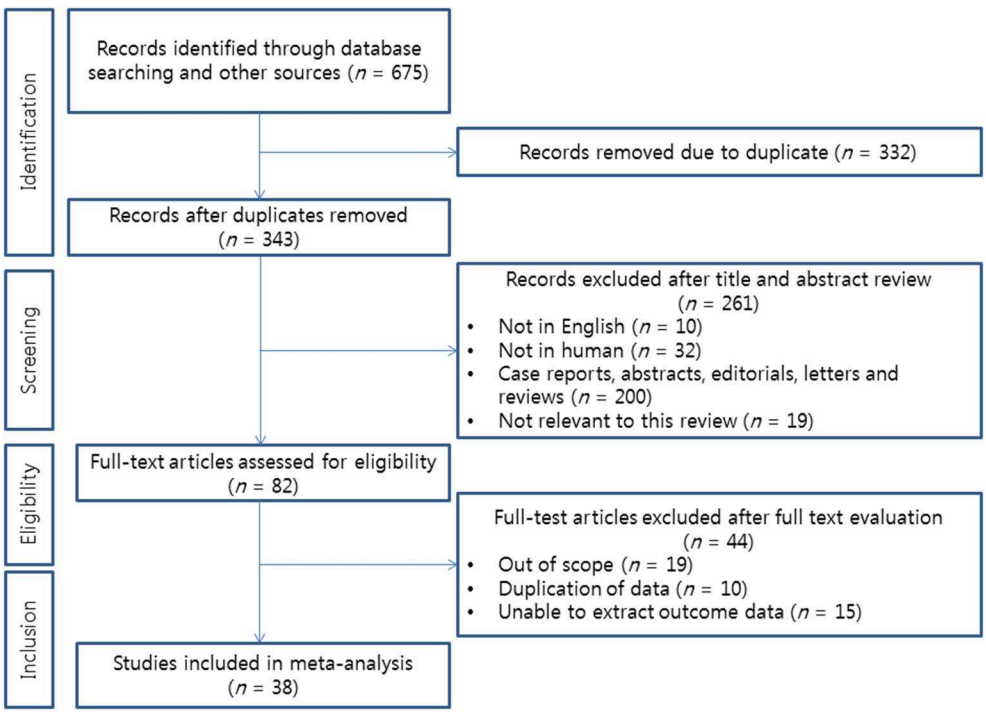

FIGURE 2 | Flow chart of the literature search for this meta-analysis.

two investigators, 82 articles remained eligible for assessment. Full-text reviews were performed by the two investigators. Finally, 38 studies were selected for meta-analysis (**Figure 2**), including seven articles on TURBT (6, 7, 15–19), 15 on radical cystectomy (8–12, 20–28), five on chemotherapy (29–33), and 11 on nephroureterectomy for upper ureter urothelial carcinoma (34–44).

Study Characteristics

The characteristics of the selected 38 studies are summarized in **Tables 1–4**. Seven articles evaluated the clinical significance of NLR in TURBT patients. Only two studies were eligible for assessing the link between NLR and invasiveness in TURBT patients (6, 7). One study performed in Italy was prospectively designed (16). Others were retrospective studies. The majority of those studies were conducted in Turkey (7, 17, 19). However, a study from Japan had the largest study population (15). The cutoff value for NLR varied from 2.2 to 3.89 depending on the study (**Table 1**). Fifteen articles were eligible for a meta-analysis of radical cystectomies (**Table 2**). Only extravesical extension, lymph node positivity, and downstaging after NAC had sufficient information to evaluate the correlation between preoperative NLR and disease status. Regarding the parameters of oncological outcomes, cancer-specific survival (CSS), overall survival, and progression-free survival (PRS) had sufficient data for linkage analysis with preoperative NLRs. Most studies set cutoff values for NLR from 2.3 to 5, however, three studies used it as a continuous variable (12, 23, 25). The studies were conducted in many countries. Two large multi-center retrospective studies were performed in the USA (10) and Europe (11). Five articles were eligible for correlation analysis of pretreatment NLRs with oncologic outcomes in chemotherapy patients (**Table 3**). All studies provided information for overall survival. However, only two studies (31, 32) provided progression-free survival data. European, American, and East Asian (Japan, China) data were included in this meta-analysis. The cutoff values for the NLR ranged from 3 to 5. One study conducted a retrospective analysis from 10 prospective phase II clinical trials (29). Eleven studies were eligible for meta-analysis of NLR association with oncological outcomes in nephroureterectomy patients (**Table 4**). Eight studies provided enough information to analyze the relationship between preoperative NLRs and PFS (34, 36, 39–42, 44, 46) and CSS (34, 36, 39–42, 44, 46). Most of those studies were conducted in East Asia. Two studies contained information on Western population patients (34, 43). All studies used NLR as a discrete variable, with cutoff values ranging from 2.0 to 3.22.

Clinical Significance of NLR in TURBT Patients

Correlation of NLR With Clinicopathologic Feature (Invasiveness)

Only two studies reported the correlation between preoperative NLRs and muscle invasiveness. The pooled OR was 4.27 (95% CI: 1.51–27.31) and moderate level of inter-study heterogeneity was present ($I^2 = 58\%$, $p = 0.12$) (**Figure 3A**). Publication bias was not assessable owing to the limited number of studies.

TABLE 1 | Characteristics of studies eligible for TURBT analysis.

Study	Country	Publication year	Recruitment period	Number patients	Study design	Inclusion and exclusion criteria	NLR cutoff value	Eligible for correlation analysis with clinicopathologic features	Eligible for correlation analysis with oncological outcomes	The Newcastle-Ottawa Scale	Quality of study
Lee et al. (6)	Korea	2015	2011–2013	226	Retrospective	Yes	3.89	Invasiveness	No	7	High
Can et al. (7)	Turkey	2012	2001–2011	182	Retrospective	Yes	2.57	Invasiveness	No	6	Moderate
Ozyalvacli et al. (17)	Turkey	2015	2008–2013	166	Retrospective	Yes	2.43	No	RFS, PFS	7	High
Mano et al. (18)	Israel	2015	2003–2010	91	Retrospective	Yes	2.41	No	RFS, PFS	7	High
Favilla et al. (16)	Italia	2016	2008–2014	178	Prospective	Yes	3	No	RFS, PFS	9	High
Ogihara et al. (15)	Japan	2016	1995–2013	605	Retrospective	Yes	2.2	No	RFS, PFS	9	High
Camtosun et al. (19)	Turkey	2017	2007–2014	89	Retrospective	Yes	2.5	No	RFS	5	Moderate

NLR, neutrophil to lymphocyte ratio; TURBT, transurethral resection of bladder tumor; RFS, recurrence-free survival; PFS, progression-free survival.

TABLE 2 | Characteristics of studies eligible for radical cystectomy analysis.

Study	Country	Publication year	Recruitment period	Number of patients	Study design	Inclusion and exclusion criteria	NLR cutoff value	Eligible for correlation analysis with pathologic status	Eligible for correlation analysis with oncological outcomes	The Newcastle-Ottawa Scale	Quality of study
Krane et al. (8)	USA	2013	2005–2011	68	Retrospective	Yes	2.5	Extravesical invasion	CSS, OS	8	High
Potretzke et al. (9)	USA	2014	2002–2012	102	Retrospective	Yes	Median 4.33*	Extravesical invasion	No	9	High
Viers et al. (10)	USA	2014	1994–2005	899	Retrospective	Yes	2.7	Extravesical invasion, LN positivity	CSS, OS	9	High
D'Andrea et al. (11)	Europe	2017	1990–2012	4198	Retrospective	Yes	3.5	Extravesical invasion, LN positivity	CSS, OS, PFS	9	High
Seah et al. (20)	Canada	2015	2006–2013	26	Retrospective	Yes	Median 2.3†	Downstaging after NAC	No	9	High
Buisan et al. (25)	Spain	2017	2007–2015	75	Retrospective	Yes	As continuous variable	Downstaging after NAC	No	9	High
Nguyen et al. (23)	USA	2016	2001–2015	310	Retrospective	Yes	As continuous variable	No	CSS, OS, PFS	7	High
Morizawa et al. (26)	Japan	2016	2002–2013	110	Retrospective	Yes	2.6	No	CSS, OS, PFS	8	High
Bhindi et al. (27)	Canada	2016	1992–2012	418	Retrospective	Yes	Median 2.9	No	CSS, OS, PFS	8	High
Ku et al. (21)	Korea	2015	1999–2011	419	Retrospective	Yes	5	No	CSS, OS	9	High
Yoshida et al. (24)	Japan	2016	1995–2014	323	Retrospective	Yes	2.7	No	OS	9	High
Ojerholm et al. (12)	USA	2017	1987–1998	230	Prospective cohort‡	Yes	As continuous variable	No	OS	8	High
Kawahara et al. (45)	Japan	2016	1999–2014	74	Retrospective	Yes	2.38	No	OS	7	High
Gondo et al. (22)	Japan	2012	2000–2009	189	Retrospective	Yes	2.5	No	CSS	8	High
Ozcan et al. (28)	Turkey	2015	1990–2013	363	Retrospective	Yes	2.5	No	CSS	7	High

Calculated cutoff value for upstage to non-organ confined disease in this study.

†*Median pre-NAC NLR value was used for this meta-analysis.*

‡*The study was a secondary planned analysis from a SWOG-8710 prospective cohort.*

NLR, neutrophil to lymphocyte ratio; CSS, cancer-specific survival; OS, overall survival; PFS, progression-free survival; NAC, neoadjuvant chemotherapy.

TABLE 3 | Characteristics of studies eligible for chemotherapy analysis.

Study	Country	Publication year	Recruitment period	Number of patients	Study design	Inclusion and exclusion criteria	NLR cutoff value	Eligible for correlation analysis with oncological outcomes	The Newcastle-Ottawa Scale	Quality of study
Taguchi et al. (30)	Japan	2015	2003–2011	200	Retrospective	Yes	3	OS	9	High
Rossi et al. (31)	Europe	2015	2003–2012	292	Retrospective	Yes	3	OS, PFS	9	High
Sonpavde et al. (29)	Multi-region (USA, Europe, Canada)	2016	2000–2016	708	Retrospective*	Yes	5	OS	8	High
Auvary et al. (32)	Europe (France, Turkey)	2016	2002–2014	208	Retrospective	Yes	3.2	OS, PFS	9	High
Su et al. (33)	China	2017	1997–2014	256	Retrospective	Yes	3.0	OS	9	High

**This study retrospectively reviewed 10 phase II prospective trials.*
NLR, neutrophil to lymphocyte ratio; OS, overall survival; PFS, progression free survival.

TABLE 4 | Characteristics of studies eligible for nephroureterectomy analysis.

Study	Country	Publication year	Recruitment period	Number of patients	Study design	Inclusion and exclusion criteria	NLR cutoff value	Eligible for correlation analysis with oncological outcomes	The Newcastle-Ottawa Scale	Quality of study
Azuma et al. (44)	Japan	2016	1998–2008	137	Retrospective	Yes	2.5	CSS, PFS	9	High
Tanaka et al. (41)	Japan	2014	1993–2011	665	Retrospective	Yes	3.0	CSS, PFS	9	High
Luo et al. (42)	China	2014	2004–2010	234	Retrospective	Yes	3.0	CSS, PFS	9	High
Kim et al. (39)†	Korea	2015	1999–2010	277	Retrospective	Yes	5.0	CSS, PFS	9	High
Sung et al. (40)	Korea	2015	1994–2011	410	Retrospective	Yes	2.5	PFS	9	High
Song et al. (36)	China	2016	2005–2011	140	Retrospective	Yes	2.2	PFS	9	High
Ito et al. (46)	Japan	2016	1999–2013	71	Retrospective	Yes	2.0	PFS	9	High
Vartolomei et al. (34)	Multi-region (USA, Europe, Canada)	2017	1990–2008	2477	Retrospective	Yes	2.7	PFS, CSS	9	High
Dalpiaz et al. (43)	Europe	2014	1990–2012	202	Retrospective	Yes	2.7	CSS, OS	9	High
Huang et al. (35)	China	2016	2002–2013	481	Retrospective	Yes	3.22	CSS, OS	9	High
Cheng et al. (38)	Taiwan	2016	2005–2010	420	Retrospective	Yes	2.7	CSS, OS	9	High

† This study provided two values for NLR, the first one was the actual NLR from each neutrophil and lymphocyte count. The second one was a derived NLR, which was calculated from: neutrophil count/(white blood cell count – neutrophil count). The two NLR values had different cutoff values. We used the actual NLRs for consistency.
NLR, neutrophil to lymphocyte ratio; OS, overall survival; PFS, progression free survival; CSS, cancer specific survival.

Correlation of NLR With Oncological Outcomes (Recurrence, Progression)

Five studies were eligible for assessing the relationship between NLRs and RFS (15–19). The results from the meta-analysis of RFS showed a negative association between high NLRs and RFS. The pooled HR was 2.32 (95% CI: 1.77–3.05). Heterogeneity was not present ($I^2 = 20\%$, $p = 0.28$) (**Figure 3B**). PFS was analyzed using data from four studies (15–18). NLRs above the cutoff value of each study were associated with a higher probability of recurrence (pooled HR: 2.45, 95% CI: 1.49–4.02). Inter-study heterogeneity was not found ($I^2 = 0\%$, $p = 0.63$) (**Figure 3C**). Publication bias was not founded in either meta-analysis.

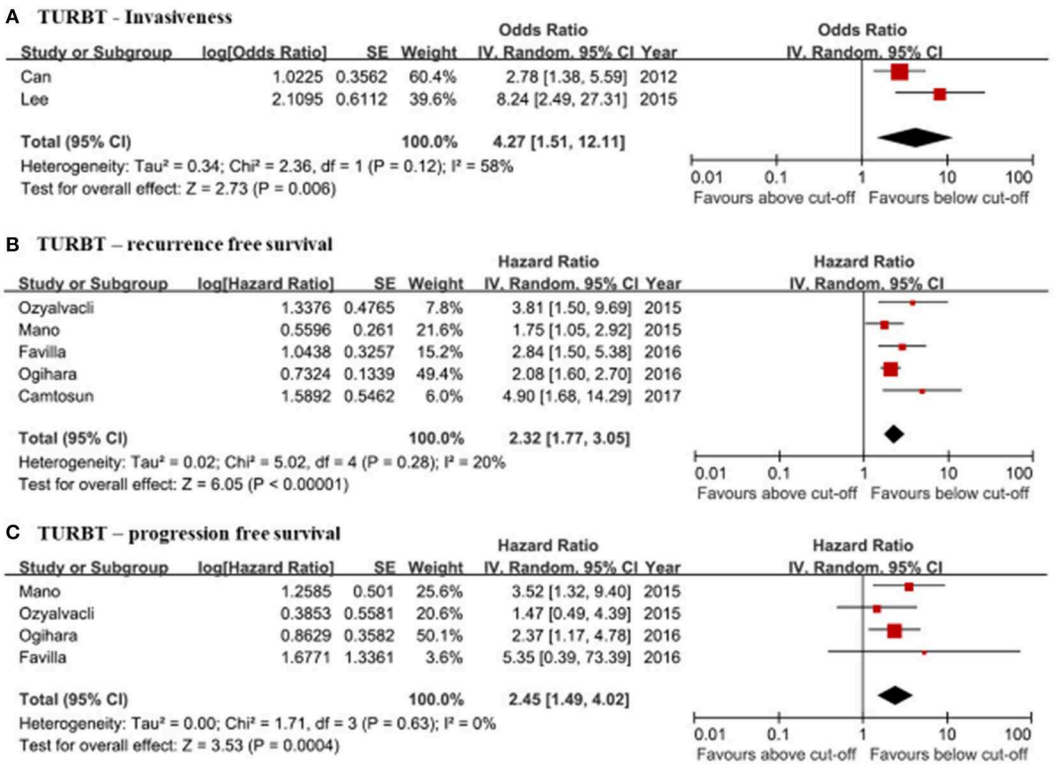

FIGURE 3 | Forrest plots of the relationship between neutrophil-to-lymphocyte ratios (NLR) and clinical information of the TURBT patients. **(A)** TURBT—Invasiveness. **(B)** TURBT—recurrence free survival. **(C)** TURBT—progression free survival.

Clinical Significance of NLR in Radical Cystectomy Patients

Correlation of NLR With Clinicopathologic Features (Extravesical Extension, Lymph Node Positivity) and Prediction of Clinical Response (Downstaging After Neoadjuvant Chemotherapy)

In four studies (8–11), extravesical extension was evaluated for pathologic up-staging to analyze the correlation with preoperative NLRs. The pooled OR was 1.14 (95% CI: 0.91–1.43) and inter-study heterogeneity was present (I^2 = 72%, p = 0.01) (**Figure 4A**). The correlation between NLRs and lymph node (LN) positivity was assessed in two large, retrospective studies (10, 11). The pooled OR was 1.43 (95% CI: 0.83–2.46). Heterogeneity between the two studies was highly present (I^2 = 97%, $p < 0.00001$) (**Figure 4B**). Pathologic downstaging after neoadjuvant chemotherapy was evaluated in two studies (20, 25). The pooled OR was calculated to be 0.79 (95% CI: 0.64–0.99). Heterogeneity between the two studies was not present (I^2 = 0%, p = 0.67) (**Figure 4C**). Publication biases could not be assessed due to the limited number of studies for LN positivity and pathologic downstaging after NAC. Publication bias was not found in the meta-analysis of extravesical extension.

Correlation of NLR With Oncological Outcomes (Overall Survival, Cancer-Specific Survival, Progression-Free Survival)

PFS was evaluated by meta-analysis in five studies (10, 11, 23, 26, 27). The pooled hazard ratio was 1.12 (95% CI: 1.03–1.31). There was a high inter-study heterogeneity (I^2 = 82%, p = 0.0002) (**Figure 4D**). Ten studies were eligible for assessing pooled HRs of the NLRs associated with overall survival in radical cystectomy patients (8, 10–12, 21, 23, 24, 26, 27, 45). The pooled HR from meta-analysis was 1.18 (95% CI: 1.08–1.30). There was high inter-study heterogeneity (I^2 = 84%, $p < 0.00001$) (**Figure 4E**). Nine studies had qualified data for evaluating the correlation of NLR with cancer-specific survival (8, 10, 11, 21–23, 26–28). The pooled HR was 1.35 (95% CI: 1.18–1.55). There was high heterogeneity (I^2 = 88%, $p < 0.00001$) (**Figure 4F**). Publication bias could not be excluded for OS, CSS, or PFS by inverted funnel plots. Not publishing negative results was suspected.

Clinical Significance of NLR in Chemotherapy Patients

Correlation of NLR With Oncological Outcomes (Progression-Free Survival, Overall Survival)

The pooled analysis of OS was based on five studies (29–33). Pretreatment NLRs were significantly associated with OS (HR: 1.44, 95% CI: 1.28–1.62) (**Figure 5A**). Only two studies reported information on PFS (31, 32). The pooled hazard ratio of PFS in these studies was 1.30 (95% CI: 1.02–1.64) (**Figure 5B**). Inter-study heterogeneity was moderately present in the meta-analyses of OS (I^2 = 30%, p = 0.22) and PFS (I^2 = 64%, p = 0.09). Publication bias was not shown by funnel plots. However, the number of studies for PFS was too small to accurately evaluate.

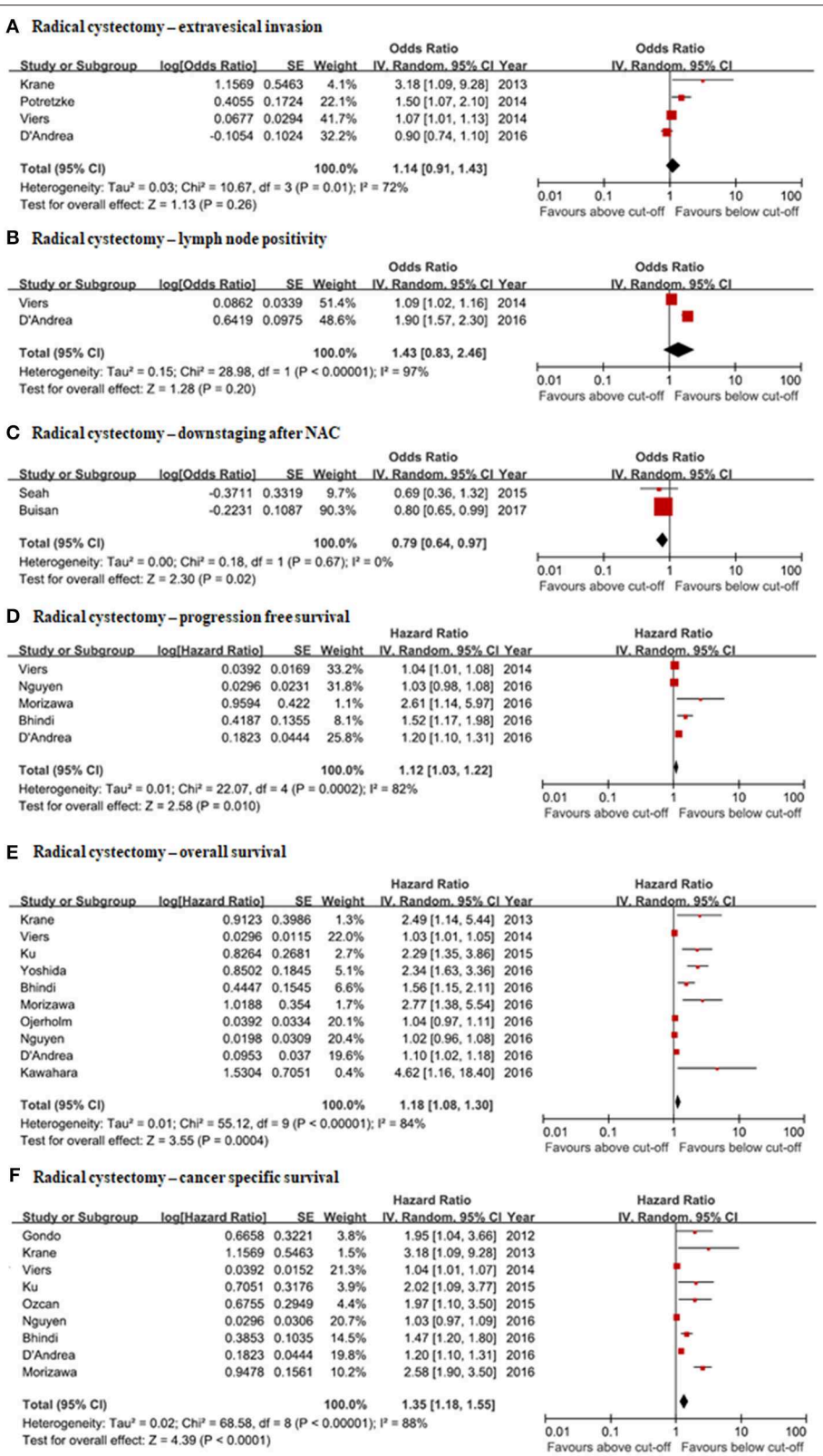

FIGURE 4 | Forrest plots of the relationship between NLRs and clinical information of radical cystectomy patients. **(A)** Radical cystectomy—extravesical invasion. **(B)** Radical cystectomy—lymph node positivity. **(C)** Radical cystectomy—downstaging after NAC. **(D)** Radical cystectomy—progression free survival. **(E)** Radical cystectomy—overall survival. **(F)** Radical cystectomy—cancer specific survival.

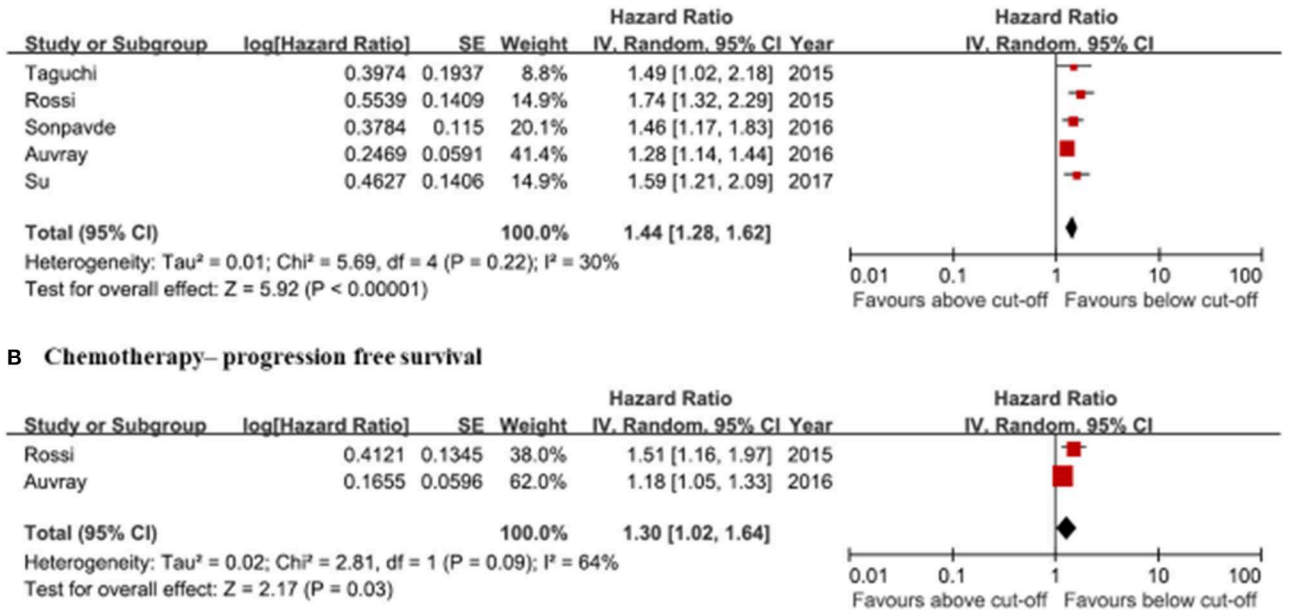

FIGURE 5 | Forrest plots of the relationship between NLRs and clinical information in chemotherapy patients. **(A)** Chemotherapy—overall survival. **(B)** Chemotherapy—progression free survival.

Clinical Significance of NLR in Nephroureterectomy Patients

Correlation of NLR With Oncological Outcomes (Progression-Free Survival, Overall Survival, Cancer-Specific Survival)

Pooled hazard ratios were calculated for PFS, OS, and CSS in this meta-analysis. A total of eight studies reported the relationship between preoperative NLRs and PFS. Preoperative NLRs were significantly associated with PFS (HR: 1.63, 95% CI: 1.22–2.18) (**Figure 6A**). Preoperative NLRs lower than the cut-off value were also associated with better CSS (HR: 1.68, 95% CI: 1.23–2.31) (**Figure 6B**). The HR of preoperative NLR for overall survival in nephroureterectomy patients was 1.72 (95% CI: 1.31–2.25) based on the meta-analysis results of three eligible studies (**Figure 6C**). Heterogeneity among these enrolled studies was moderately present for CSS and PFS but was not present for OS. There was a risk of publication bias with a possible risk of not reporting negative results.

DISCUSSION

The neutrophil-to-lymphocyte ratio (NLR) is one of the most actively studied biomarkers for predicting disease status in various cancer types (4, 47). The clinical evidence for the usefulness of NLR as a biomarker in urothelial carcinoma has been accumulating over the last several years, but the topic is still under debate. We conducted a systematic review and meta-analysis to assess the correlation between pretreatment NLRs with pathologic features and the prognosis of urothelial carcinoma patients. This study collected a total of 38 studies containing clinical information and oncological outcomes on 16,379 patients. To our knowledge, this is the largest and latest meta-analysis evaluating the clinical significance of pretreatment NLRs for each specific urothelial carcinoma management situation.

Our evidence supports pretreatment NLR as a useful biomarker for assessing disease aggressiveness and oncological outcomes in TURBT, radical cystectomy, chemotherapy, and nephroureterectomy patients. High NLR was associated with muscle invasiveness (OR: 4.27), poor RFS (HR: 2.32), and PFS (HR: 2.45) in TURBT patients. The major focus of transurethral management of superficial bladder urothelial carcinoma is controlling recurrence and progression to muscle-invasive disease. If bladder cancer presents with muscle-invasion and a high risk of progression or recurrence, guidelines recommend radical cystectomy (48, 49). The TURBT procedure always has the risk of failing to obtain the detrusor muscle in the specimen, which is important for proper staging and disease management (50). For this reason, repeated TURBTs should be considered in high-risk bladder NMIBC. The pretreatment NLR before TURBT could provide additional information for selecting the proper treatment strategies for the management of bladder urothelial carcinoma. High NLR values were associated with a higher chance of extravesical extension (OR: 1.14), lymph-node positivity (OR: 1.43), and worse oncological outcomes. The pooled hazard ratios for PFS, CSS, and overall survival were 1.2, 1.35, 1.18, respectively. Patients with low pretreatment NLRs showed better response rates after NAC followed by radical cystectomy, with a higher chance of pathologic down-staging

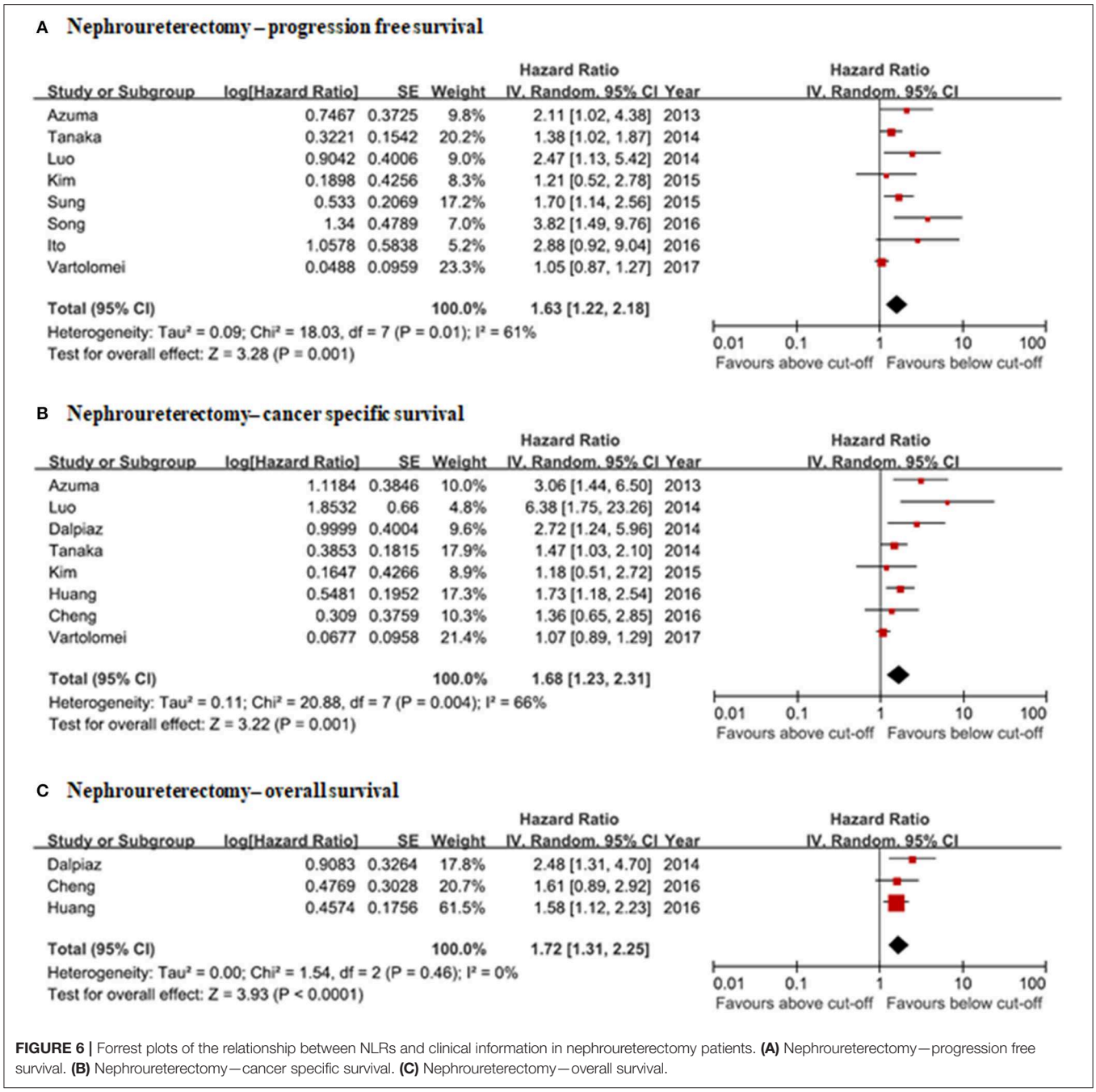

FIGURE 6 | Forrest plots of the relationship between NLRs and clinical information in nephroureterectomy patients. **(A)** Nephroureterectomy—progression free survival. **(B)** Nephroureterectomy—cancer specific survival. **(C)** Nephroureterectomy—overall survival.

(OR: 0.79). The oncological outcomes of chemotherapy and nephroureterectomy also revealed negative correlations with pretreatment NLRs. We presumed that the correlation between high pretreatment NLRs and poor outcomes after treatment for urothelial carcinoma was a result of more aggressive presentations, however, further well-designed studies are needed to clarify this hypothesis.

Although results from many studies favor NLR as a useful biomarker under many clinical situations, its predictive mechanism remains unclear. We generally accepted that there is a correlation between inflammation and cancer, however, a causal relationship is ambiguous. Chronic inflammation caused by infection or toxic materials leads to tumorigenesis in many solid tumors. About 20% of tumors are associated with prior viral, microbial, and parasite infections, including infections with the hepatitis virus, human papillomavirus, *Helicobacter pylori,* and *Schistosoma haematobium* (51). Additionally, recent studies showed an inverse correlation between non-steroid anti-inflammatory drugs (NSAID) and cancer risk (52). Inflammatory cells are recruited to the tumor

microenvironment in the situation of tumor advancement with invasion or the distant migration of tumor cells (53). The cancer-related immune response is paradoxical. Molecular evidence has demonstrated that tumor-infiltrated inflammatory cells eradicate nascent tumors (54). However, some studies have shown that increased systemic inflammation enhanced tumor development, progression, and metastasis (55). Because of the unclear mechanism of the NLR to predict disease prognosis, the clinical utility of the NLR is very limited. Moreover, we have only limited evidence for the association of NLR and prognosis compared to important prognostic factors, such as pathologic stage and surgical margins. Thus, a targeted study evaluating the prognostic impact of NLR after adjusting for covariable factors is needed.

The definition of an optimum NLR value is another problem. In studies included in this meta-analysis, the cutoff value of NLR varied from 2.0 to 5.0 and some studies even used NLR as a continuous variable (12, 23, 25). In addition, NLR is a dynamic marker of the systemic immune response. Thus, we could not judge the optimal cutoff value easily. NLR is not only related to bladder cancer but also related to many benign and malignant diseases. Therefore, it can be increased without the advancement of tumors. Recently, some studies used a derived NLR introduced by Proctor et al. (56) that makes the clinical utility of NLR more complex.

This systematic review and meta-analysis had some limitations. First, most studies on the relationship of NLR with many clinical parameters and oncological outcomes were retrospectively designed. There was no prospective, randomized controlled study that met our search criteria. Variable differences in study design, patient numbers, the definition of oncological outcomes, and NLR cutoff values also affected the inter-study heterogeneity. Publication bias was another limitation, especially in the sub-group analysis of cystectomy, and nephroureterectomy patients. In addition, we could not include immune-oncological agent targeted studies, which are promising treatments for advanced urothelial carcinoma and thought to be strongly correlated with systemic immune responses. Finally, this meta-analysis used articles written in English only. Thus, we could not exclude language bias in the favorable positive results. Despite these limitations, this study-level meta-analysis provided a generalized view of NLRs on disease aggressiveness, oncological outcomes, and treatment responses in patients with urothelial carcinoma.

CONCLUSION

This study-level meta-analysis showed that pretreatment NLRs were useful biomarkers for disease aggressiveness, oncological outcomes, and treatment responses in the management of urothelial carcinoma. However, inter-study heterogeneity, the possibility of publication bias, the limited number of eligible studies, and no randomized controlled study were limitations of the study. A large, well-designed, prospective study is needed to provide clear evidence that the pretreatment NLR is a useful biomarker for urothelial carcinoma.

AUTHOR CONTRIBUTIONS

JK: conception and design. CJ, CK, HK, and JK: collection and assembly of data. JS: manuscript writing. All authors data analysis and interpretation, final approval of manuscript, and accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01365/full#supplementary-material

Supplementary Data 1 | Funnel plots of each analysis.

Supplementary Data 2 | New Castle Ottawa Scale Summary: The review authors' judgments on each parameter for each included study.

Supplementary Data 3 | The New Castle Ottawa Scale detailed review of each study.

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The Rare Variant rs35356162 in *UHRF1BP1* Increases Bladder Cancer Risk in Han Chinese Population

Junlong Wu[1,2†], *Meilin Wang*[3†], *Haitao Chen*[4,5†], *Jianfeng Xu*[5,6†], *Guiming Zhang*[7], *Chengyuan Gu*[1,2], *Qiang Ding*[5], *Qingyi Wei*[8,9], *Yao Zhu*[1,2*] *and Dingwei Ye*[1,2*]

[1] Department of Urology, Fudan University Shanghai Cancer Center, Shanghai, China, [2] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China, [3] Department of Genetic Toxicology, The Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing, China, [4] State Key Laboratory of Organ Failure Research, Guangdong Key Laboratory of Viral Hepatitis Research, Department of Infectious Diseases and Hepatology Unit, Nanfang Hospital, Southern Medical University, Guangzhou, China, [5] Fudan Institute of Urology, Huashan Hospital, Fudan University, Shanghai, China, [6] Center for Cancer Genomics, Wake Forest School of Medicine, Winston-Salem, NC, United States, [7] Department of Urology, The Affiliated Hospital of Qingdao University, Qingdao, China, [8] Cancer Institute, Fudan University Shanghai Cancer Center, Shanghai, China, [9] Duke Cancer Institute, Duke University Medical Center, Durham, NC, United States

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***Correspondence:**
Yao Zhu
mailzhuyao@163.com
Dingwei Ye
dingwei_ye1963@163.com

†These authors have contributed equally to this work

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Background: Seventeen loci have been found to be associated with bladder cancer risk by genome-wide association studies (GWAS) in European population. However, little is known about contribution of low-frequency and rare variants to bladder cancer susceptibility, especially in Eastern population.

Methods: We performed a three-stage case-control study including 3,399 bladder cancer patients and 4,647 controls to identify low-frequency and rare variants associated with bladder cancer risk in Han Chinese. We examined exome-array data in 1,019 bladder cancer patients and 1,008 controls in discovery stage. Two replication stages were included to validate variants identified. Bonferroni adjustment was performed to define statistical significance. Logistic regression was conducted to evaluate single marker association with bladder cancer risk. We used SKAT-O method to perform gene level-based analysis. We also conduct additional experiments to explore the underlying mechanism of filtered gene(s).

Results: We identified a novel rare coding variant (rs35356162 in *UHRF1BP1*: G > T, OR = 4.332, $P = 3.62E\text{-}07 < 7.93E\text{-}07$, Bonferroni cutoff) that increased bladder cancer risk in Han Chinese. Gene-level analysis showed a significant association of *UHRF1BP1* ($P = 4.47E\text{-}03$) with bladder cancer risk. Experiments indicated down-regulation of *UHRF1BP1* promoted migration and invasion through epithelial-mesenchymal transition in bladder cancer cell lines.

Conclusion: The rare variant of *UHRF1BP1*, rs35356162, increases bladder cancer risk in Han Chinese and UHRF1BP1 might act as a tumor suppressor in bladder cancer development and progression.

Summary: Little is known about potential contribution of low-frequency and rare variants to bladder cancer susceptibility. We performed a three-stage case-control study and identified a new rare variant, rs35356162 in *UHRF1BP1*, which increased bladder cancer risk in Han Chinese.

Keywords: bladder cancer, *UHRF1BP1*, exome array, Chinese, single nucleotide variant

INTRODUCTION

Bladder cancer is the 7th most common cancer globally (1) and ranks 1st in urologic malignancies in China (2). Bladder cancer incidence varies among different geographic regions, owing to different genetic background, lifestyles and environmental factors (3–5). Cigarette smoking and occupational exposure to aromatic amine compounds are two well-known risk factors (6), while genetic predisposition factors may explain one-third of all the bladder cancer cases (7).

Genome-wide association studies (GWAS) have identified 17 independent loci and single nucleotide variants (SNVs) that contribute to bladder cancer susceptibility in European population [(8–14); **Table S1**]. Many loci have been replicated in additional studies, and additional new loci found to be, associated with bladder cancer risk in Chinese population (15–21). GWA studies have been successful in identifying common variants involved in complex trait etiology. However, SNVs identified by GWAS are common variants with a minor allele frequency (MAF) over 5%, which only had small individual effect sizes with an odds ratio (OR) ranging from 1.1 to 1.5 mostly. Thus, the "missing heritability" remains an issue in interpreting GWAS results (22). It was reported that < 10% of genetic variance could be explained by common variants via GWAS for the majority of complex traits (23). Additionally, low-frequency ($1\% \leq$ MAF $\leq 5\%$) and rare (MAF $< 1\%$) variants were not included in modern GWAS chips, which could lead to missing heritability as well.

To date, many low-frequency and rare variants affecting the risk of complex traits have been found, such as rare coding mutations of *BRIP1* and *RAD51D* in ovarian cancer (24, 25), and rare variants of *ATM*, *RAD50,* and *PALB2* in breast cancer (26). However, little is known about the contribution of low-frequency and rare variants to bladder cancer risk. Therefore, we performed a three-stage study, aiming to identify new low-frequency or rare variants that are associated with bladder cancer risk in Han Chinese. Functions of the relevant gene in bladder cancer were also explored.

MATERIALS AND METHODS

Study Population and Design

This study is a three-stage case-control study. The discovery stage included 1,019 bladder cancer cases and 1,008 controls to filter variants associated with BCa risk. In replication I and II stages, a total of 2,404 BCa cases and 3,639 controls were recruited to validate variants accordingly. Detailed recruitment criteria and study design supplements was summarized in **Supplementary Materials and Methods**. All individuals recruited in this study were unrelated Han Chinese people.

Slides of BCa cases were confirmed by two pathologists independently and results were reported based on the 2004 WHO/ISUP classification criteria. Clinical characteristics were collected via medical records. Cancer history, symptom and smoking status was collected from medical records or phone inquiry. A smoking status of "Yes" represented current smokers at diagnosis or those who had ever smoked daily for over 1 year before diagnosis.

This study was performed according to the ethical standards of the Helsinki Declaration II and approved by the Scientific and Ethical Committee of Fudan University Shanghai Cancer Center and other Institutional Review Board of participating hospitals. Informed consents were obtained from all subjects.

Exome Array Genotyping and Calling

We performed exome array genotyping using Illumina HumanExome-12 v1.1 beadchip (see URL: Exome Chip Wiki) in 1,019 bladder cancer cases and 1,008 controls in the discovery stage. Genotype calling was carried out by standard Illumina's GenTrain version 2.0 clustering algorithm using GenomeStudio software (V2011.1). Cluster boundaries were determined using Illumina's standard cluster file. The datasets used and/or analyzed during the current study are available at https://doi.org/10.17632/bkvnsfgd4y.1.

Exome Chip Analysis

To select proper SNVs for further analysis, we conducted quality control of samples and SNPs according to the procedures described in **Supplementary Materials and Methods**. Finally, 20 cases failed IBS analysis (**Figure S1**), 3 cases were duplicated samples and 1 case had incomplete clinical data. Notably, 636 SNVs failed HWE test in control group. So after quality control of samples and SNVs, a total of 995 cases and 1,008 controls with 63,047 SNVs remained (Details of quality control in **Tables S2**, **S3**). Because we shared the same controls with ChinaPCa project, these 1,008 controls survived same filtering procedures before (27). Principal component analysis (PCA) was performed using EIGENSOFT. A set of SNVs that showed low linkage disequilibrium (LD; $r^2 < 0.1$) were used to estimate population outliers in a principal component analysis. The result was shown in **Figure S2**.

Selection of Variants in Replication Stages

SNVs detected in discovery stage were classified into three categories: common variants, low-frequency, and rare variants, and reported variants previously. These three kinds of variants

were filtered following different procedures (**Tables S4–S6**). *P*-value thresholds for selection were presented as follows: 0.001 for common variants, 0.01 for low-frequency and rare variants and 0.05 for variants in previous reported GWAS data. Twenty-six SNVs were selected for validation in replication I stage using Sequenom MassARRAY. Based on the combined results from discovery stage and replication I stage, validation was performed in replication II stage using TaqMan probes (Life Technology, Carlsbad, CA, USA). Results were analyzed using SDS2.4 software (Applied Biosystems, Foster City, CA, USA). All genotyping was conducted independently by technicians in a blinded manner. Detailed filtering procedures were shown in **Supplementary Materials and Methods**.

Functional Experiments

Additional experiments were performed to explore functions of certain gene(s) selected from three-stage study in bladder cancer development and progression. Detailed description of cell lines and culture, plasmids construction and lentivirus preparation, RNA extraction, reverse transcription and quantitative real-time PCR analysis, antibodies for western blot, cell cycle assay, cell proliferation, migration and invasion assays were presented in **Supplementary Materials and Methods**.

Statistical Analysis

We performed univariate logistic regression analysis without adjustments of clinical features to calculate odds ratio (OR) and 95% confidence interval (CI) to estimate association between single variant and bladder cancer risk in an additive model. If no polymorphism was detected in control group, single-variant association analysis was performed using Fisher exact test. Hardy-Weinberg Equilibrium was compared using Pearson's Chi-square test. An identity-by-state similarity score was obtained using PLINK (see URLs). For gene-level analysis, we conducted the sequence kernel association optimal (SKAT-O) test, using reference Gene file from UCSC. The SKAT-O test included all the SNVs which survived filtering procedures, 63,047 SNVs in total. Statistical analysis and plotting were mainly carried out using R software (see URLs) and PLINK. In addition, Bonferroni adjustment was used for three stages combined to find out significant variant which was associated with bladder cancer risk. The Bonferroni cutoff was calculated as 0.05/63,047 SNVs, which meant the significance boundary was 7.93E-07.

PolyPhen2 (see URLs) was used to predict the functional impact of certain variant based on sequence and structure's predictive methods. Amino acid conservation analysis was based on multiple sequences alignment performed on Vector NTI 11.5.1 (Invitrogen, Carlsbad, CA, USA) and was plotted using CTex (see URLs).

RESULTS

Exome Array Analysis

Demographics of the participants in this three-stage study are shown in **Table 1**. The flow chart of our study design and primary results are summarized in **Figure 1**.

In the discovery stage, 995 bladder cancer cases and 1,008 controls were qualified for subsequent analysis. The

TABLE 1 | Characteristics of subjects analyzed in the discovery and subsequent replication stages.

Characteristics	No. (%)					
	Discovery stage		Replication I stage		Replication II stage	
	Cases (*N* = 995)	Controls (*N* = 1,008)	Cases (*N* = 1,156)	Controls (*N* = 1,273)	Cases (*N* = 1,248)	Controls (*N* = 2,366)
Age, years						
Mean (SD)	65.1 (14.5)	61.5 (9.5)	64.5 (12.2)	63.6 (11.2)	64.9 (12.5)	58.7 (12.6)
Median (Range)	63.0 (27.0–89.0)	62.0 (41.0–79.0)	64.0 (26.0–96.0)	65.0 (27.0–90.0)	66.0 (16.0–96.0)	60.0 (28.0–89.0)
Gender						
Male	799 (80.3)	1,008 (100)	930 (80.5)	816 (64.1)	1,010 (80.9)	1,501 (63.4)
Female	196 (19.7)		226 (19.5)	457 (35.9)	238 (19.1)	865 (36.6)
Smoking status						
Never	617 (62.0)	455 (45.1)	632 (54.7)	882 (69.3)	573 (45.9)	1,613 (68.2)
Ever	370 (37.2)	507 (50.3)	473 (40.9)	384 (30.2)	470 (37.7)	742 (31.4)
Unclear	8 (0.8)	46 (4.6)	51 (4.4)	7 (0.5)	205 (16.4)	11 (0.4)
Grade						
Low grade	373 (37.5)		302 (26.1)		491 (39.4)	
High grade	551 (55.4)		288 (24.9)		673 (53.9)	
Other	71 (7.1)		566 (49.0)		84 (6.7)	
Stage						
Non-muscle invasive	735 (73.9)		689 (59.6)		784 (62.8)	
Muscle invasive	245 (24.6)		251 (21.7)		418 (33.5)	
Other	15 (1.5)		216 (18.7)		46 (3.7)	

principal component analyses revealed that cases and controls were genetically matched (**Figure S2**). And, as shown in the quantile-quantile plot (**Figure S3**), the inflation factor was 0.98. A representative cluster plot (rs35356162) generated by GenomeStudio was presented in **Figure S4**. Cluster plots for all SNVs in this study can be obtained from online available source data (https://doi.org/10.17632/bkvnsfgd4y.1). We determined the association of single variant with bladder cancer risk according to the following three categories: common variants, low-frequency and rare variants, and variants based

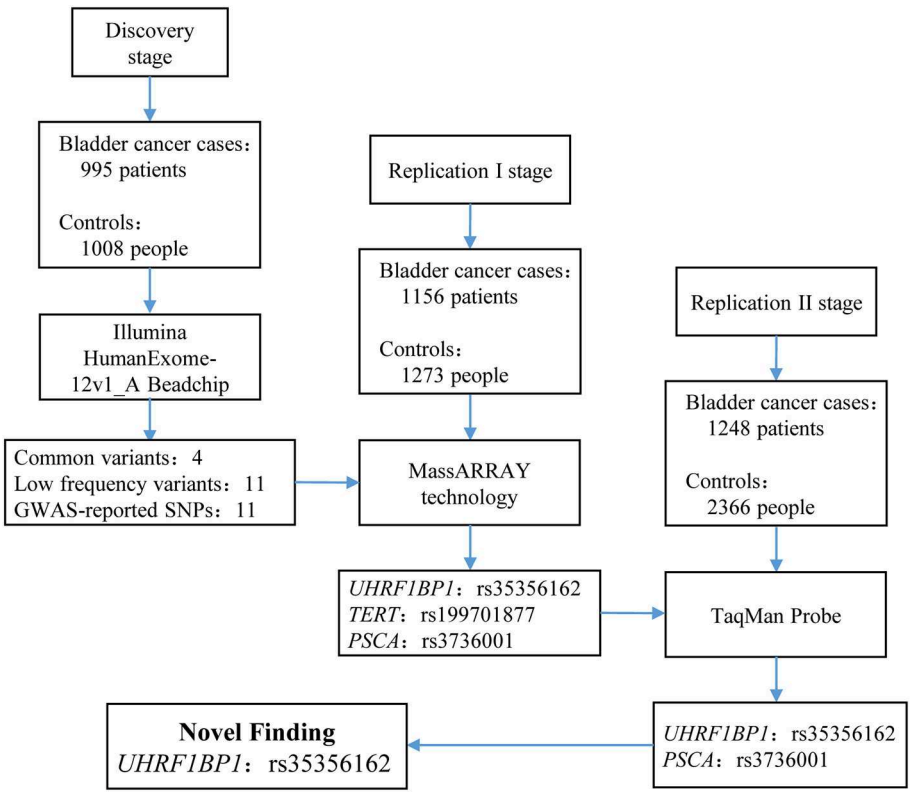

FIGURE 1 | Flow chart of study design and summary of the three-stage study. In discovery stage, we enrolled 995 bladder cancer cases and 1,008 controls to perform exome chip analysis. After filtering, 26 SNVs remained and entered into replication I stage for further validation via MassARRAY technology. In replication I stage, only 3 SNPs survived. These three SNPs entered into replication II stage for validation. Finally, only rs35356162 in *UHRF1BP1* and rs3736001 in *PSCA* survived. However, only rs35356162 in *UHRF1BP1* achieved statistical significance after Bonferroni adjustment. More study design supplements and aim of each stage can be found in **Supplementary Materials and Methods**.

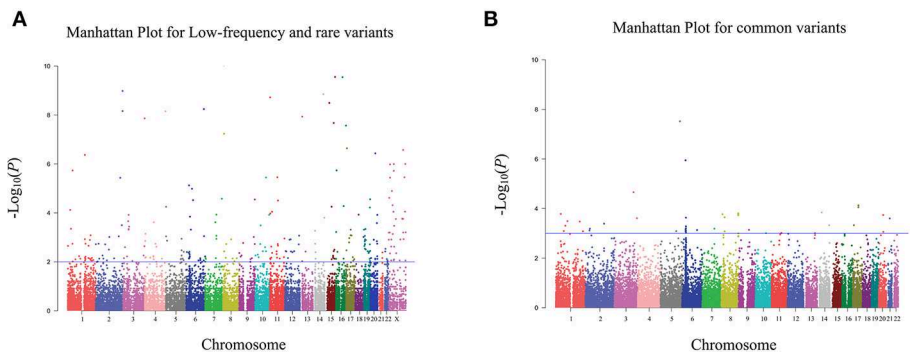

FIGURE 2 | Manhattan plot for exome chip of bladder cancer in Han Chinese populations in the discovery stage. **(A)** Manhattan plot for low-frequency and rare variants. **(B)** Manhattan plot for common variants. The X axis represents the chromosomal position and the Y axis represents the $-\log_{10}$ *P*-value. The blue line indicates the filtering threshold of $-\log_{10}$ *P*-value. For low-frequency and rare variants, the blue line represented 0.01. For common variants, the blue line represented 0.001.

on previously reported GWAS results. Manhattan plots for common variants, low-frequency, and rare variants are shown in **Figure 2**, with a line representing primary filtering threshold *P*-value. Different screening procedures were conducted between common and low-frequency and rare variants (**Tables S4**–**S6**). After filtering, we identified 4 common variants and 11 low-frequency and rare variants that were significantly associated with bladder cancer risk. In addition, we also genotyped additional 11 SNVs that were in previous reported GWA studies (21). Finally, 26 SNVs were selected for further validation in the additional cohorts. Details of these variants are presented in **Table S8**.

Validation of Selected Variants in the Replication Stages

To evaluate the 26 SNVs selected in the discovery stage, they were genotyped in an independent replication I cohort of 1,156 cases and 1,273 controls using Sequenom MassArray Technology (**Table 1**). In this stage, only 3 SNVs achieved a $P < 0.05$, including a rare variant rs35356162 in the UHRF1 binding protein 1 gene (*UHRF1BP1*), and two previously reported variants: rs199701877 in the telomerase reverse transcriptase gene (*TERT*) and rs3736001 in the prostate stem cell antigen gene (*PSCA*). Genotyping details of these three SNVs are summarized in **Table S9**.

In the replication II stage, three SNVs, which survived in the replication I stage, were further validated in a cohort including 1,248 BCa cases and 2,366 cancer-free controls. Only *UHRF1BP1* rs35356162 and *PSCA* rs3736001 survived all three stages (**Table S10**). However, combined analysis after Bonferroni adjustment showed that only *UHRF1BP1* rs35356162 (OR = 4.332, 95% CI: 2.463 – 7.619, $P = 3.62E-07 < 7.93E-07$) was identified as independent variant significantly associated with BCa risk in Han Chinese (**Table 2**).

Gene-Level Based Test

Considering the majority of individual variants were low-frequency or rare variants, and the limited power of single marker association analysis, we performed the SKAT-O test to evaluate the gene-level test as recommended (28). For the two variants passed the three-step filtering, the SKAT-O analysis based on exome chip genotyping demonstrated significant associations of *UHRF1BP1* ($P = 4.47E-03$) and *PSCA* ($P = 1.30E-03$) with BCa risk (**Table 2**). We also listed top 10 genes (with at least 10 SNVs in SKAT-O test) that were predicted to be highly associated with BCa risk in discovery stage, based on exome array results (**Table S11**).

Population Genetics and Functional Prediction

Population genetics of rs35356162 based on 1,000 Genomes Project Phase 3 showed that MAF of rs35356162 was 0.0012 globally, while a higher MAF (0.003) was observed in Eastern Asian population. The T allele frequencies of rs35356162 in Chinese Han Beijing population (0.0049) and Chinese Han south

TABLE 2 | Summary of association with bladder cancer risk for rs35356162 and rs3736001 in the three-step stages and gene-based analysis of these two genes.

SNV	Gene	Variant	Locus	Minor/Major allele	Stage	Genotypes*		MAF		OR (95% CI)	*P*-Value	*P*-Value (SKAT-O test)	Number of SNVs in SKAT-O test
						Cases	Controls	Cases	Controls				
rs35356162	*UHRF1BP1*	p. Gly152Val	6p21.31	T/G	Discovery	0/21/974	0/3/1,000	0.0106	0.0015	7.187 (2.137–24.172)	1.44E-03	4.47E-03	13
					Replication I	0/16/1,116	0/5/1,255	0.0071	0.0020	3.599 (1.314–9.855)	1.27E-02		
					Replication II	0/13/1,235	0/8/2,354	0.0052	0.0017	3.097 (1.280–7.493)	1.21E-02		
					Combined	**0/50/3,325**	**0/16/4609**	**0.0074**	**0.0017**	**4.332 (2.463–7.619)**	**3.62E-07**		
rs3736001	*PSCA*	p. Glu30Lys	8q24.3	A/G	Discovery	15/216/758	8/171/829	0.1244	0.0928	1.391 (1.137–1.703)	1.36E-03	1.30E-03	2
					Replication I	15/241/872	14/228/1,018	0.1201	0.1016	1.208 (1.007–1.448)	4.15E-02		
					Replication II	18/264/966	17/437/1,908	0.1202	0.0997	1.239 (1.060–1.447)	6.93E-03		
					Combined	**48/721/2,596**	**39/836/3755**	**0.1214**	**0.0987**	**1.265 (1.143–1.399)**	**5.00E-06**		

**Genotypes presented for rs35356162 in UHRF1BP1: TT/GT/GG and genotypes presented for rs3736001 in PSCA: AA/GA/GG.*

Odds ratio and P-value derives from logistic regression in additive model.

Bold values highlight the combined results of three stages.

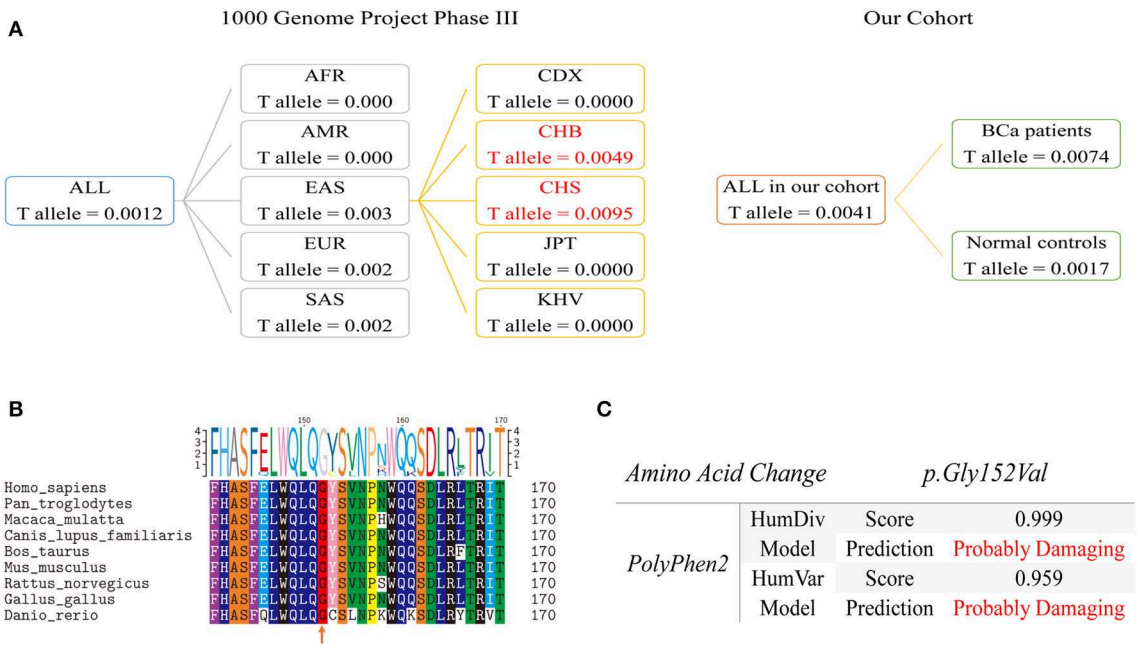

FIGURE 3 | Population genetic comparison, evolutionary conservation analysis and functional prediction. **(A)** Left Panel: T allele frequency of rs35356162 in the 1000 Genomes Project. Frequencies of mainland Han Chinese subgroup are highlighted in red. Right Panel: the T allele frequency of rs35356162 in the present study, shown separately by bladder cancer cases and controls. **(B)** UHRF1BP1 p.Gly152 amino acid conservation analysis across species assessed with multiple sequences alignment. The orange arrow indicated the amino acid position which rs35356162 may alter. **(C)** *In silico* predicted effects of p.Gly152Val variant, assessed with PolyPhen2, by HumDiv and HumVar models. HumDiv model is a preferred model for evaluating rare alleles and dense mapping of regions identified by GWAS. HumVar model is a preferred model for diagnostics of Mendelian diseases which requires distinguishing mutations with drastic effects from all the remaining human variation, including abundant mildly deleterious alleles. In this study, HumDiv model is preferred, but we also presented result from HumVar model as a reference.

population (0.0097) were much higher than that detected in our study (**Figure 3A**).

Multiple sequences alignment across species revealed that p.Gly152 was a highly conserved amino acid site during evolution, which could lead to pathogenicity if altered (**Figure 3B**). The missense variant rs35356162 (p.Gly152Val) was predicted to be probably deleterious by PolyPhen2 (**Figure 3C**). Combined predictive results above indicated that this variant (p.Gly152Val) could probably change UHRF1BP1 protein function. Predictive results from other tools were shown in **Figure S5**.

In vitro Functional Validation of *UHRF1BP1*

Functional validation was performed to determine whether UHRF1BP1 played tumor-suppressive role in bladder cancer cell lines. Western blotting showed that J82 cell line was proficient in expressing UHRF1BP1 protein (**Figure 4A**). Two different short-hairpin RNA sequences both could remarkably down-regulate the expression of UHRF1BP1 in transcriptional level and translational level (**Figures 4B,C**). Down-regulation of UHRF1BP1 could sharply increase the ability of cell invasion and cell migration in both knockdown cell lines (**Figures 4D,E**). Quantitative real-time PCR analysis was performed to compare expression level of 16 epithelial-mesenchymal transition (EMT) related genes between scramble group and sh-UHRF1BP1-B group. The results further showed that all epithelial markers were down-regulated in the knockdown group, especially *E-cadherin*, *Desmoplakin*, and *EpCAM*, while mesenchymal markers *ZEB2* and *N-cadherin* were significantly up-regulated in the knockdown group (**Figure 4F**). Western blotting of representative EMT markers confirmed the results from PCR panels above (**Figure S6**). We also assessed proliferation ability alteration and cell-cycle distribution difference among scramble and knockdown groups, and achieved consistent results (**Figure S7**).

DISCUSSION

By examining multiple coding variants in a three-stage case–control study, we were the first to find that a low frequency variant in *UHRF1BP1*, rs35356162, increased bladder cancer susceptibility in Han Chinese population. The gene-level analysis indicated *UHRF1BP1* was strongly associated with BCa incidence, and functional experiments revealed a tumor suppressive function.

UHRF1BP1 is located on chromosome 6p21 and encodes a protein with an unclear function. UHRF1BP1 was found to be an important part of the ICBP90 complex and a putative binding protein of UHRF1 in 2004 (29). Some studies demonstrated that several non-synonymous variants of *UHRF1BP1* were associated with systemic lupus erythematosus, both in European descendants and Chinese populations (30, 31). The role of UHRF1BP1 in cancer was initially investigated by a Japanese research group. They found that the interaction of

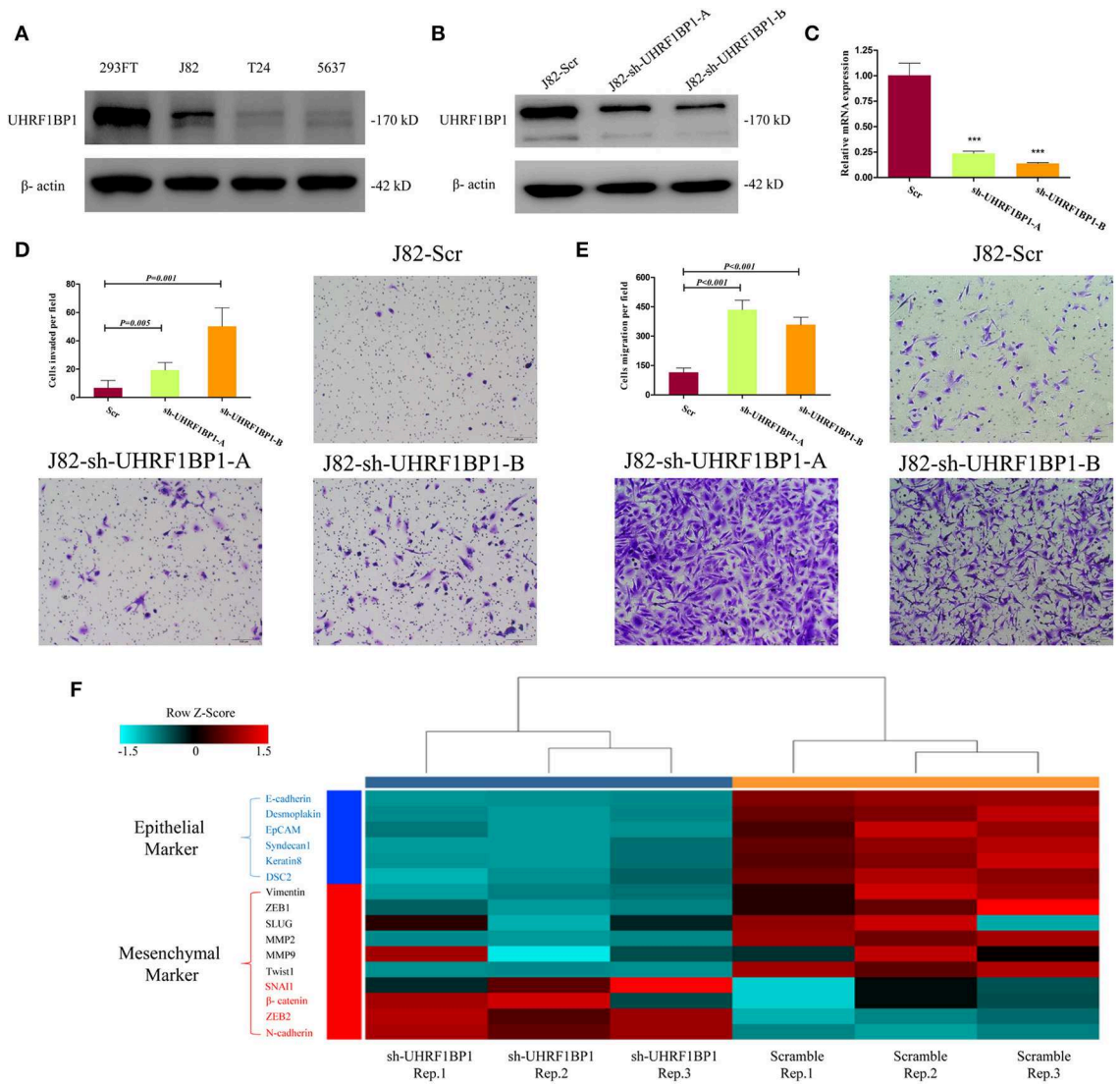

FIGURE 4 | Down-regulation of UHRF1BP1 in J82 cell line can promote cell migration and invasion. **(A)** UHRF1BP1 is relatively highly expressed in J82 bladder cancer cell line, β-actin as a loading control. HEK293FT cell line acts as a positive control. **(B)** UHRF1BP1 protein expression is significantly inhibited by sh-UHRF1BP1-A&B. **(C)** UHRF1BP1 mRNA level is significantly inhibited by sh-UHRF1BP1-A&B. **(D)**. Representative images of invasion assay for J82 cells infected by scramble and sh-UHRF1BP1-A/B lentiviruses. **(E)** Representative images of migration assay for J82 cells infected by scramble and sh-UHRF1BP1-A/B lentiviruses. **(F)** Heatmap shows the mRNA expression differences of 16 genes involved in EMT between J82-Scr cells and J82-sh-UHRF1BP1-B cells in triplicates. This PCR array includes six epithelial markers (blue) and 10 mesenchymal markers (red). ***$P < 0.001$

UHRF1 with UHRF1BP1 may lead to relocation of UHRF1, while overexpression of UHRF1BP1 appeared to inhibit cell growth in colon cancer cell lines (29), which indicated that UHRF1BP1 could act as a tumor suppressor. Results in our study are consistent with previous deduction. Down-regulation of UHRF1BP1 expression in bladder cancer cell lines promoted invasion and migration, probably through EMT. Down-regulation of UHRF1BP1 expression can also promote cell proliferation, but perhaps not by regulating cell cycle. Hence, further studies should explore detailed mechanisms of tumor suppression ability, especially the interaction between UHRF1BP1 and UHRF1, which may play important roles in tumor DNA methylation transferring and other epigenetic events (32–36).

Population genetic comparison showed that the T allele frequency of rs35356161 in our study cohorts was lower than that in CHB or CHS cohort in the 1,000 Genomes Project. As described, the CHB population was collected in Beijing surrounding areas and the CHS population was recruited in Hunan and Fujian Provinces (37). However, participants of the present study were collected in Yangtze River Delta. The frequency difference may reflect heterogeneity of genetic background in Han Chinese population during the migration and fusion of nationalities in Chinese history, to some extent.

Previous GWAS studies reported that *PSCA* rs2294008 and rs2978974 conferred susceptibility to BCa in Caucasians and that the T allele of rs2294008 was associated with increased *PSCA* mRNA expression in both BCa tissues and normal bladder tissues (9, 38). Wang et al. validated the association between rs2294008 and increased BCa risk in a Han Chinese population (16). In our study, we observed that another *PSCA* variant, rs3736001, might also increase BCa risk, although this variant did not reach statistical significance. Given the obvious importance of *PSCA* polymorphisms in bladder cancer, functional studies of larger samples are warranted to delineate the precise effect of PSCA on bladder cancer.

Although the discovery stage had a relatively small sample size and limited statistical power, based on our calculation, we could also have a 0.98 power to detect the SNV with an OR of 4 and a frequency of 0.01 in control. A major strength of the present study is a large collection of Han Chinese case-control studies including a total of 3,399 bladder cancer patients and 4,647 controls in a three-stage study design, which ensures reliability of the results. However, there are certainly some limitations. Firstly, there was no female individual in the control group of discovery stage. This may lead to a selection bias. Secondly, apart from principal component analysis and clinical ethnic information collection, we did not perform other analyses to test genetic consistency in our study. This may reduce the reliability of the association study if without following multi-stage replication and functional analysis. Thirdly, we only performed basic functional evaluation of UHRF1BP1 in bladder cancer cell lines without mechanism exploration. Single amino acid-mutated plasmid and wild-type plasmid of UHRF1BP1 should have been constructed to do more precise investigation to further understand the role of UHRF1BP1 in cancer biological process. Finally, we did not collect detailed clinical and pathological data well, such as TNM staging, tumor multifocality and carcinoma *in situ*. We only focused on cancer risk correlation and missed the association between clinicopathological factors and SNVs, and also reduced the feasibility and reliability of subgroup analysis. Because of many missing data in clinical and pathological information, we only performed univariate logistic regression analysis to estimate the association between single variant and bladder cancer risk in Chinese population, without adjustment of clinicopathological features.

In conclusion, we found that a rare variant of *UHRF1BP1*, rs35356162, could increase bladder cancer risk in Han Chinese population. Our findings highlight the value of low-frequency and rare variants in identifying BCa associated genetic variation and cast a new light in BCa epidemiological screening and prevention.

URL

R Statistical software: http://www.R-project.org/;
Illumina: http://www.illumina.com/;
Exome chip design: http://genome.sph.umich.edu/wiki/Exome_Chip_Design/;
PLINK: http://pngu.mgh.harvard.edu/~purcell/plink/;
EIGENSOFT: http://genepath.med.harvard.edu/ reich/Software.htm; PolyPhen2: http://genetics.bwh.harvard.edu/pph2/;
CTex: http://www.ctex.org/HomePage/;
ChinaPCa: http://www.chinapca.org.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in Mendeley Data, https://doi.org/10.17632/bkvnsfgd4y.1.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Scientific and Ethical Committee of Fudan University Shanghai Cancer Center with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Scientific and Ethical Committee of Fudan University Shanghai Cancer Center.

AUTHOR CONTRIBUTIONS

DY and YZ conceived and designed the research. JW, MW, and JX acquired the data. JW drafted the manuscript. JW and GZ performed experiments. HC and CG analyzed the data. QD and QW contributed reagents, materials, and analysis tools.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00134/full#supplementary-material

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PERSPECTIVE
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How Do We Meet the Supportive Care and Information Needs of Those Living With and Beyond Bladder Cancer?

Sara Jane MacLennan and Steven MacLennan*

Academic Urology Unit, University of Aberdeen, Aberdeen, United Kingdom

This perspective paper presents the case for adopting a new approach to the design and delivery of supportive care for those with bladder cancer. It is our assertion that the design and delivery of supportive care for those diagnosed with bladder cancer needs to (1) build on existing research and available tools and (2) address current limitations due to lack of use of said tools, lack of understanding of research and needs, lack of a shared language, and method of assessment and evaluation. This, we argue, can be achieved through a network-based approach (1) focussed on the structure, process, and outcome of supportive care.

Keywords: bladder cancer, supportive care, information, unmet needs, quality of life

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****Correspondence:***
Sara Jane MacLennan
s.maclennan@abdn.ac.uk

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INTRODUCTION

There is growing agreement across all tumor types that we need to treat the disease-condition and to meet the information and supportive care needs of those living with and beyond cancer; to support individuals to have a life lived well (2). A diagnosis of bladder cancer (non-muscle invasive and muscle-invasive) is a stressful life event with numerous supportive care needs that continue beyond initial treatment and yet there is a lack of research around informational and supportive care needs of this group (3). Quality of life and unmet needs have not been well-researched across the whole illness trajectory which in part is due to a lack of high quality measurement instruments that are consistently used. This perspective paper acknowledges the need for better information and supportive care across the cancer journey and outlines a vision for how this might be achieved.

Over the past 5 years, there have been a handful of studies that have attempted to describe or map the information and supportive care needs of those diagnosed with bladder cancer from the perspective of the individual (3–8). There are few studies which have looked at the understanding of these needs from the other main stakeholder groups (health care professionals (e.g., urologists, oncologists, cancer nurse specialists), researchers and non-profit organizations) in the design and delivery of care.

The majority of studies have focused on unmet needs around aspects of (1) the experience of bladder cancer and treatment (such as living with a urostomy or sexual function following cystectomy), (2) quality of life and domains of functioning (such as cognitive, social, sexual, and emotional) and (3) experience of and satisfaction with care (support, information, continuity, burden, and inconvenience). A small number of these have attempted to map these needs across the cancer journey. Some studies conclude that reported needs appear to be being met through current care systems and delivery; others that there remain large gaps in both our understanding and in closing the gap between research and the design and delivery of care. It is difficult to draw

substantive conclusions across these studies due to the heterogeneity of the research; studies around mapping the unmet needs of those diagnosed with bladder cancer have measured slightly different dimensions in different ways for different groups of individuals [different types of bladder cancer: non-muscle invasive (NMIBC) and muscle-invasive (MIBC)] at different stages in their cancer journey. Examples of key studies are given below and are organized by (1) NMIBC, (2) MIBC, and (3) changing needs across the cancer journey. This is not intended to be a systematic review of the literature but is provided to allow the reader insight in to the current knowledge landscape.

Focusing on NMIBC, Rutherford et al. (9) developed a framework to describe the experience of living with and beyond a diagnosis of NMIBC. This included three key domains—(1) the disease-condition (symptoms and treatment including blood in urine; frequency, urgency, incontinence; pain when urinating, pelvic pain; nausea, vomiting, constipation, diarrhea; fatigue, loss of sleep; infection, fever; skin rashes), (2) three dimensions of functioning (cognitive, sexual functioning, emotional functioning) and (3) experiences and satisfaction with care (including support, information, continuity, burden, and inconvenience.

Focusing on MIBC, Mohamed et al. (8) explored informational (information support) and supportive care needs (medical, psychological, and emotional support) across the illness trajectory. They found that individuals reported unmet needs across five key domains—(1) health system and information needs, (2) patient care and support, (3) physical/daily living, (4) psychological well-being, and (5) sexuality. Mohamed et al. (5) then conducted a further investigation of this data and found that the unmet needs of those living with MIBC vary by age, sex, and treatment choice. They make the argument that assessment and intervention needs to be tailored to these specific needs. An argument supported by Bhanvadia (7) who also found that needs differ along racial, gender, and socio-economic groups. They too highlighted the importance of long-term support and survivorship resources and for tailored models that address quality of life and supportive care needs across the patient journey.

Paterson et al. (5) systematically reviewed and summarized the literature on supportive care needs of those with MIBC. Their paper characterizes supportive care needs in nine domains: (1) patient-clinician communication, (2) daily living needs, (3) health system/information needs, (4) practical needs, (5) family-related needs, (6) social needs, (7) psychological needs, (8) physical needs, and (9) intimacy needs. They reported that individuals with MIBC expressed high unmet needs at diagnosis and these continued beyond primary treatment. The paper acknowledged that understanding of how needs mapped across the cancer journey is still needed.

Focusing on changing needs across the cancer journey, Edmonson et al. (7) and Chung et al. (3) assessed information and supportive care needs across the illness trajectory. Both studies highlighted changes in quality of life and supportive care needs over time and argued for the need for further research (3) and better measurement of key outcomes across the cancer journey (7).

Edmonson et al. (7), through an in-depth review of the qualitative literature, mapped the lived experience and needs of those with NMIBC and MIBC on to the individual's cancer journey at diagnosis, during acute care and treatment, post-treatment, and beyond (which they name as "the new normal"). This is the first in-depth systematic review of the qualitative evidence in this area. This paper allows greater insight in to the lived experience of bladder cancer and changes in supportive care needs over time. Edmonson et al. (7) clearly highlight the need for further and better quality research in this neglected area.

Chung et al. (3) looked at quality of life, informational and supportive care needs of individuals with NMIBC and MIBC across the illness trajectory. The key supportive care needs reported were about (1) sex life; (2) decisions about life in uncertainty; (3) coping with others not acknowledging impact of cancer; (4) coping with expectations of individual as cancer survivor; (5) coping with change to belief that nothing bad will happen in life; (6) developing new relationships after cancer; (7) understanding financial entitlements; (8) accessible hospital parking; (9) impact on relationship with partner; and (10) life/travel insurance. They described these in terms of existential care needs. Most of the reported information needs were in the medical domain (knowledge of cancer, treatment options, side effects, subsequent post-treatment tests). Encouragingly, they found that individuals reported most of the identified supportive care needs had been met.

Despite these studies, gaps still exist within our knowledge of how needs change over time. Less is known about how we translate research in to practice and best meet these needs at different points across the cancer pathway (3, 7), particularly the further we move from primary treatment. We can all identify strong examples of existing good clinical practice but these are grounded in the expertise of the different teams in the different geographical locations in meeting the supportive care and information needs of their patient groups. The question becomes how do we ensure that this is the typical experience for all those diagnosed with bladder cancer within the UK, and globally?

In this perspective paper, we argue that there is a need to think more broadly in terms of the involvement and role of stakeholders in understanding supportive care needs and changing behavior. This would take the form of a multi-stakeholder approach that (1) builds a community of expertise, (2) is grounded in a pathway perspective and (3) allows the individual to be an active participant in the design and delivery of supportive care and appropriate information.

The current view of the authors is based on an extensive programme of work over the past 10 years from the University of Aberdeen to better understand the information and supportive care needs of those diagnosed with all forms of urological cancer (including bladder cancer) across the cancer journey. The focus of this has been on structure, process (key stakeholder behavior and interaction) and outcome (measurement and definition) of cancer care (10). This has included co-design work looking to address supportive care and information needs of those

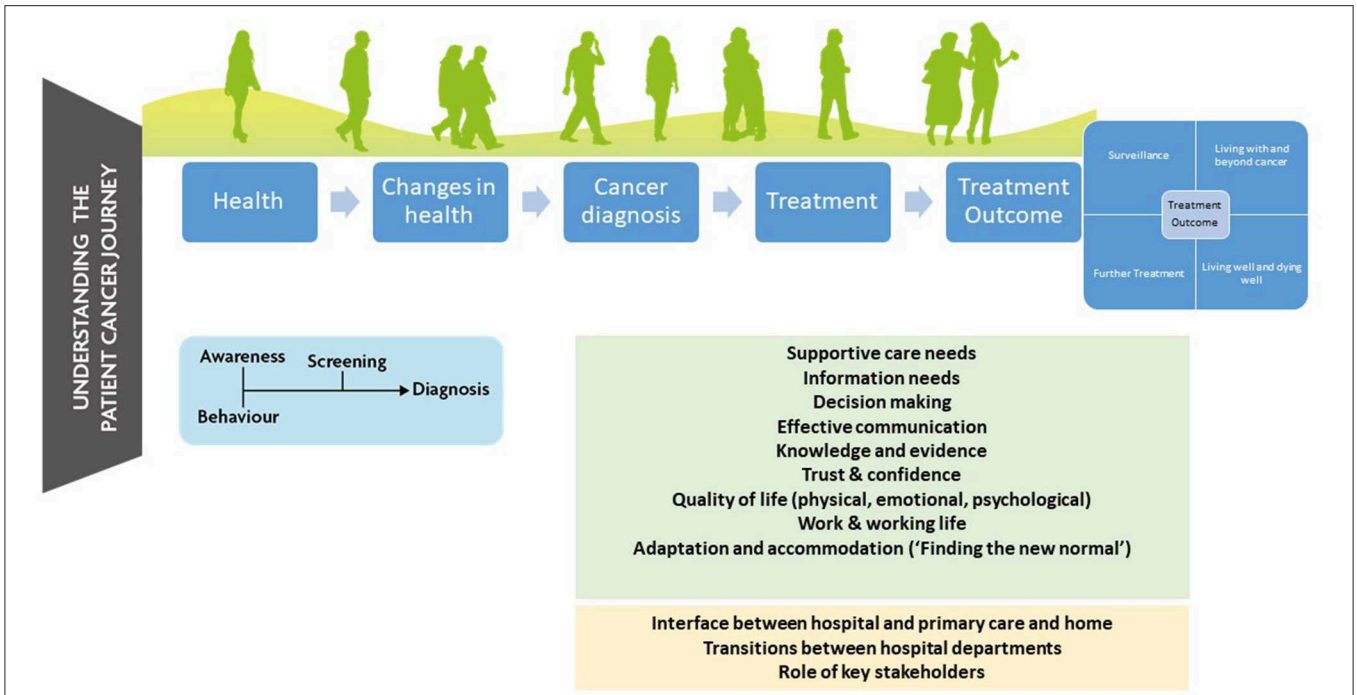

FIGURE 1 | Mapping the supportive care and information needs of those diagnosed with cancer.

diagnosed with urological cancer (11), understanding how best to include the individual's voice in the design and delivery of care, and the language and measurement of care and outcomes e.g., clinical guidelines and core outcome sets [For more detail on individual projects see (1, 12–18)] (see **Figure 1**).

This has culminated in an understanding that the design and delivery of supportive care and timely and appropriate information is necessarily more complex than a simple allocation of responsibility for providing this care to one stakeholder group or role or individual. This responsibility has to be shared across stakeholder groups across the time line of the person's journey from diagnosis to decision making to treatment to follow-up and continuing forward. Essentially, a network-based approach (1) to the design and delivery of care grounded in understanding *the process* of these interactions between stakeholder groups.

A network-based approach requires a number of things to be successful. First, it requires the main stakeholder groups to understand each other's roles, priorities, expertise, level of knowledge and behavioral drivers. This would be achieved through characterizing knowledge, attitudes, and the determinants of the key behaviors [e.g., using the theoretical domains framework (19)]. Second, it requires that each stakeholder group is confident in their own knowledge and the accepted boundaries of their own competence. They also need to be confident in the knowledge and competence of those who they can signpost to within this process. Third, it requires better, more informed communication between individuals as well as within and between stakeholder groups which is supported by appropriate structures and a reliable method to measure this (e.g., are treatment and care achieving the outcomes that matter most to the key stakeholder groups?). Step two and three build on step one and require the identification of behavior change techniques to change clinical practice and improve uptake of evidence in to practice [e.g., COM-B systems of behavior change (20)].

If we accept the need for a network-based approach (*process*), this also has to be supported by education and training. First, education is required in terms of the nature and current availability of reliable and useful information about bladder cancer and treatment and how best to tailor this to the individual's diagnosis and cancer journey to support shared-decision making. Second, training and informed discussion are required in terms of appropriate ways of delivering that information and when. Third, training and informed discussion are required to support the development of local networks and role profiles and an appropriate structure to achieve this. Such education and training could be provided by one of the major independent actors in this area, for example, Fight Bladder Cancer, Macmillan Cancer Support.

The need for *appropriate structures* of care is important in achieving the aim of better supportive care and information. There is an argument for building on existing structures to include behavior and the interactions between the main stakeholder groups. This also needs to keep pace with developments in diagnosis and treatment (e.g., personalized medicine, big data and prognostic and predictive biomarkers). This could take many forms but would have three key functions: (1) to collect information from the individual, (2) to

process in real-time and to deliver appropriate and evidence-based information back to the individual and (3) act as a record of these "conversations" for future reference and for review.

Examples of existing structures include the recognition of information and supportive care needs as a priority within clinical practice guidelines for bladder cancer [e.g., (21)] and the availability of tools and systems to (1) assess healthcare needs, (2) provide access to information and resources, and (3) to inform recommendations for healthcare teams and for individuals (care plan and treatment summary) [e.g., the National Cancer Survivorship Initiative and Macmillan's Recovery Package and the online Holistic Needs Assessment tool (eHNA) through mycareplan (2)].

In an ideal world, every individual would have access to a platform that would collect and collate PROMs in real-time and flag information and supportive care needs to the healthcare team and the individual across the cancer journey. This would support shared decision-making and person-centered care. This would also act as a platform to capture the information and supportive care delivered and to map communication with the capacity to "learn" over time. Existence of such a record would also encourage reflection on advice given, support consistency in that advice and facilitate better communication. This record would also be a summary of the different stakeholder roles in the process.

The important point to highlight about *structure* is that it needs to be informed by *process* and *outcome*. The development of new online and evidence-based tools to provide information such as those flagged are necessary but not sufficient to fully address the problem. In addition to the development of such evidence-based tools, we need to understand the process in which these are used and useful and the outcomes that are being measured and reported.

We have outlined our proposal for two essential elements in the design and delivery of supportive care and information (*process* and *appropriate structures*). The final element in this is *outcomes*. A move toward the design and delivery of evidence-based supportive care needs to be matched by better measurement and the inclusion of core outcomes as trial end points and in big data. When we try to overview all the evidence that has been reported for the available treatments for bladder cancer, we often find that it is difficult to compare, contrast and summarize it. A main reason for this is heterogeneity in outcome reporting and definitions. That is, many studies comparing the same interventions in the same patient populations use different outcomes or define the same outcomes in different ways. This in turn makes it difficult to make recommendations for treatment in clinical guidelines and it hampers decision making for clinicians and patients. A solution to this is to create core outcome sets (COS). A COS is an agreed standardized collection of outcomes which should be measured and reported, as a minimum, in all trials for a specific clinical area (22). These may be extended to data collection in routine practice, as proposed by ICHOM (23).

A focus of COS development is that patients should be involved as key stakeholders in prioritizing which outcomes are most important to them and thereby ought to be measured in future trials or day to day clinical practice. Work is ongoing to develop a COS for the various stages of bladder cancer (http://www.comet-initiative.org/studies/details/1135). This will assess to what extent currently available measures are fit for purpose and if new tools need to be developed which better reflect patient's concerns. In future, when the most important outcomes are consistently measured in the same ways across trials, it will be easier facilitate treatment decision-making and monitor patient's outcomes across the cancer journey so that timely intervention may be prompted. This links back to the processes and systems we have already discussed.

It is our firm belief that the design and delivery of supportive care for those diagnosed with bladder cancer needs to (1) build on existing research and available tools (e.g., celebrate success and not reinvent the wheel and (2) address current limitations due to lack of use of existing tools, lack of understanding of existing research and examples of best practice, lack of a shared language, and method of assessment and evaluation. This can be achieved through a network-based approach focussed on the structure, process and outcome of supportive care.

DISCUSSION

In conclusion, there is a very real need to continue progress and build on successes in this area to better support those living with bladder cancer. The intention behind this paper is to act as a nudge to researchers and healthcare professionals working within this area to commit to action and drive change. We can achieve this by working to deliver research in to the design and delivery of information and supportive care for bladder cancer that is grounded in a network-based approach. We need to be able to bring together research identifying the unmet needs of those diagnosed with NMIBC and MIBC and core outcomes, current clinical practice and excellent supportive care and strong information resources championed by non-profits orgs such as Fight Bladder Cancer. This should also inform policy and healthcare planning, the commissioning of resources and clinical practice guidelines. As we have proposed during this brief paper, there is a clear role for the key stakeholder groups (individuals living with and beyond bladder cancer, health care professionals (urologists, oncologists, cancer nurse specialists, primary care), researchers and non-profit organizations) to come together to innovate and communicate. The solution lies in working together. What is needed now is action.

AUTHOR CONTRIBUTIONS

SJM and SM contributed substantively to the development of the perspective described within the manuscript and the preparation of the manuscript.

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Back-Splicing Transcript Isoforms (Circular RNAs) Affect Biologically Relevant Pathways and Offer an Additional Layer of Information to Stratify NMIBC Patients

Anshita Goel[1], Douglas G. Ward[1], Naheema S. Gordon[1], Ben Abbotts[1], Maurice P. Zeegers[2], K. K. Cheng[3], Nicholas D. James[1], Richard T. Bryan[1] and Roland Arnold[1]**

[1] Institute of Cancer and Genomic Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, [2] NUTRIM School for Nutrition and Translational Research in Metabolism & CAPHRI Care and Public Health Research Institute, Maastricht University, Maastricht, Netherlands, [3] Institute of Applied Health Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

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Edited by:
Kent William Mouw,
Dana–Farber Cancer Institute,
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Daniele Baiz,
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Momoe Itsumi,
Showa University, Japan

***Correspondence:**
Richard T. Bryan
r.t.bryan@bham.ac.uk
Roland Arnold
r.arnold.2@bham.ac.uk

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Circularized transcript isoforms due to back-splicing are increasingly being reported in different tissues types and pathological states including cancer. Since these circular RNAs (circRNAs) are more stable than linear messenger RNA their identification and profiling in tumor tissue could aid in stratifying patients and may serve as biomarkers. In this study, we have investigated the relationship between circRNA expression and tumor grade in a cohort of 58, mostly non-muscle-invasive bladder cancer patients. From 4571 circRNAs detected, we identified 157 that were significantly differentially expressed between tumor grades relative to the linear transcript. We demonstrated that such grade-related differences can be identified in an independent cohort, and that a large fraction of circRNAs can be, in principle, detected in urine. The differentially expressed circRNAs cluster into subgroups according to their co-expression, subgroups which are enriched for DNA repair, cell cycle and intracellular signaling genes. Since one proposed function of circRNAs is to interfere with gene-regulation by acting as microRNA "sponges," candidates which were differentially expressed between tumor grades were investigated for potential miRNA target sites. By investigating the circRNAs from bladder cancer related pathways we demonstrated that the expression of these pathways, the circRNAs, and their parental genes are often decoupled and do not correlate, yet that some circRNAs do not follow this tendency. The present study provides the next step for the comprehensive evaluation of this novel class of RNAs in the context of non-muscle-invasive bladder cancer. Intriguingly, despite their possible function as microRNA sponges, they potentially affect host mRNA levels at the transcriptional stage, as compared to post-transcriptional control by miRNAs. Our analysis indicates differences of their activity between bladder cancer tumor stages, and their relative expression levels may provide an additional layer of information for patient stratification.

Keywords: circular RNA, back-splicing, bladder cancer, tumor grade, NMIBC, biomarkers

INTRODUCTION

Circular RNAs (circRNAs) are a class of single-stranded closed RNA molecules, created by backsplicing events from linear mRNA (1). They were first identified ∼20 years ago, but were initially seen as artifacts of aberrant RNA splicing (2). However, more recently, numerous potential functions of circRNAs have been proposed, such as acting as miRNA sponges, modulating transcription and interacting with RNA-binding proteins (RBPs) (3). Additionally, several genome-wide studies indicate that circRNAs are evolutionary conserved across species (4). Furthermore, an increasing number of studies show that circRNAs are strongly correlated with the proliferation, apoptosis, invasion, and metastasis of human tumors, which indicates the potential of circRNAs to act as novel therapeutic targets and biomarkers (5). The use of circRNAs for the latter is especially intriguing since they are more stable than linear RNA forms, escaping degradation by those processes which are dependent on the recognition of the ends of RNA molecules.

With the help of novel bioinformatics approaches and appropriate sequencing methods, comprehensive studies of circRNA species are possible (6), allowing investigation of the landscape of circRNAs in healthy and cancerous tissues. circRNAs are generally expressed at low levels and often exhibit cell type specific and tissue specific patterns (7). Most circRNAs originate from exons of protein-coding genes and can consist of one or multiple exons; some circRNAs also arise from introns, intergenic regions, non-coding RNA (ncRNA) loci, and other portions of the genome (8). In eukaryotes, the lengths of circRNAs are heterogeneous, ranging from ∼100 to 4000 base pairs (9), and are estimated to account for as many as 0.1–10% of molecules in the transcriptome (10).

Bladder cancer (BC) is the 12th most common cancer worldwide (6th most common in males) with 430,000 new cases reported in 2012 (11). Over three-quarters of new cases are diagnosed as non-muscle-invasive bladder cancer (NMIBC: stages Ta/T1/Tis), with the remainder demonstrating invasion of tumor into the detrusor muscle (muscle-invasive bladder cancer, MIBC: stages T2+) (12). MIBC is a life-threatening disease requiring radical treatment and carrying a poor prognosis (13). Although NMIBC is not immediately life-threatening, recurrence after initial treatment is common and 10–15% of cases will progress to MIBC, necessitating burdensome and expensive long-term surveillance (14, 15). NMIBC is thus a heterogeneous disease with regard to both clinical outcomes (recurrence and progression) and underlying biology, with considerable differences in chromosomal alterations and mutational events between grades (low/high grade or grades 1–3) and stages (Ta/T1/Tis) (16). Furthermore, expression analyses derived from largescale RNA sequencing initiatives have tended to classify NMIBCs into three subtypes: the Lund Group describe urobasal A (UroA), genomically unstable (GU), and infiltrated, overlapping with the UROMOL classification of Class 1 (UroA, -progression signature, -CIS signature), Class 2 (GU, +progression signature, +CIS signature), and Class 3 (pronounced expression of lincRNAs, decreased expression of genes associated with cell cycle and metabolic processes, and increased expression of genes associate with histone modifications and chromatin remodeling) (17).

More recently, the integration of chromosomal and expression alterations with the mutational landscape has identified six molecular subtypes of BC with different molecular features, prognoses and distributions between NMIBC and MIBC (18): the Neural-like subtype is prevalent in MIBC and characterized by high WNT/β-catenin signaling; HER2-like is distributed evenly across NMIBC and MIBC, with higher *ERBB2* amplification and signaling; Papillary-like is a NMIBC subtype enriched in urothelial differentiation genes with a high frequency of actionable *FGFR3* mutations, amplifications, and *FGFR3-TACC3* fusion; Luminal-like is also predominantly NMIBC, has higher MAPK signaling and more *KRAS* and *KMT2C/D* mutations than other subtypes; Mesenchymal-like and Squamous-cell carcinoma-like are predominant in MIBC. Importantly, about 20% of NMIBCs show MIBC subtype traits and a lower 5-yr OS rate than Papillary-like NMIBC (81 vs. 96%) (18).

Genomic and epigenomic approaches may thus inform the development of more accurate risk stratifiers and non-invasive diagnostics (19), tools that are urgently required by healthcare professionals and patients alike (20). CircRNAs also appear to have a role to play in this already complex setting as a novel class of prognostic biomarkers for NMIBC—in a pioneering study, Okholm et al. (21) evaluated circRNAs in a large cohort ($n = 457$) of NMIBCs and identified circRNAs that are differently expressed between high and low risk tumors, highlighting two (circHIPK3 and circCDYL) as potential biomarkers (21).

In this study, we investigate circRNAs in a set of 58 tumors and evaluate circRNAs, demonstrating that for a subset, their relative expression is tumor grade dependent and may represent an alternative or additional molecular classification. We also provide an initial analysis of the overlap of the circRNAs detected in our study with the results of Okholm et al. in order to evaluate reproducibility between different tumor sample sets and alternative computational pipelines.

MATERIALS AND METHODS

Samples and Library Preparation for RNA-Sequencing

Fresh-frozen tumors were collected as part of the West Midlands' Bladder Cancer Prognosis Programme (BCPP). This study was carried out in accordance with the recommendations of the International Committee on Harmonization Good Clinical Practice (ICH GCP) guidelines. The protocol was approved by the NHS Health Research Authority East Midlands—Derby Research Ethics Committee (ref: 06/MRE04/65). All participants gave written informed consent for the donation of tissue biospecimens and their subsequent utilization in biomedical research. RNA sequencing was performed on RNA extracted from 58 snap frozen incident bladder cancers (urothelial carcinomas) using RNeasy mini kits (Qiagen). Sequencing libraries were prepared from total RNA using Truseq Stranded Total RNA kits with Ribo-zero Gold ribosomal RNA depletion (Illumina) and paired-end sequenced using the Illumina NextSeq

platform (2 × 75 bp). On average, 48 million reads were obtained per tumor sample.

QC and Alignment

Raw fastq files were first processed using FastQC which makes diagnostic plots; subsequently, for each read, Trimmomatic (version 0.32) (22) was used to trim two bases from the start and clip where average phred score quality fell below 20. Quality-checked fastq reads were then mapped to the human genome (version GRCh37) and Ensembl gene annotation (release 87) using STAR aligner (ver 2.5.2b) (23).

circRNA Prediction

Two different strategies were used to identify potential back-splicing events in the transcriptome which predict circular RNAs (circRNAs) to result in an *in-silico* reconstruction of circRNA coordinates. The CircExplorer2 algorithm (24) was used on the "chimeric.out.junction" file (from STAR aligner) containing information on potential non-linear alignments obtained for each sample. The second method, the DCC algorithm (25), differs from CircExplorer as it applies STAR separately on the R1 and R2 from the paired-end fastq data. To retain a high confidence data set, the output from each algorithm was filtered to select only those cases which had ≥4 reads supporting the back-splicing event in at least one of the samples.

Of the 4,571 circRNA candidates predicted by DCC, ∼66% were also detected by CircExplorer2. Since DCC also provides count data for circRNAs and counterpart linear RNAs (host gene's mRNA) to permit calculation of circular-to-linear ratio, the output of the DCC algorithm was chosen for downstream analyses.

Positional Bias of Exonic circRNAs

To identify any positional bias within the gene body, the genomic coordinates corresponding to RefSeq genes and their untranslated regions (UTRs) and coding sequences (CDS) were retrieved from UCSC Table Browser (hg19). In addition, bedtools (ver. 2.27.1) was used to overlap the exonic circRNA coordinates individually with the CDS, 5′ and 3′ UTR coordinates.

Differential Expression

For the back-splice junctions implicated, read counts were resolved into circRNA and linear RNA (junction count) components using DCC. To control for different library sizes, circular-to-linear ratios were calculated after normalizing for RNA-seq library size resulting in counts per million (CPM); relative expression values were computed by $\log_2$ [(CPM(circularRNA)+1)/(CPM(linearRNA)+1)]. Circular-to-linear ratios were used to perform Analysis of Variance (ANOVA) using R (ver. 3.5.1) to identify tumor grade discriminating circRNA candidates (at adjusted *p*-value 0.05).

Pathway Analysis

GO enrichment was carried out between a list of differentially expressed genes and the background of all parental genes of circRNAs using Gorilla (26). To compare sets of interests against the complete human background, we used the functional enrichment tool of the STRING database (version 11.0) (27). To further explore the biological processes affected by differentially expressed genes, we carried out pathway analyses using the Enrichr online tool (28). Pathways were adjudged using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database with a *p*-value threshold of <0.05.

In order to investigate whether the circRNAs showed different behavior compared to host genes in selected pathways, we selected previously-reported important bladder cancer pathways (29, 30). These include the pathways "DNA repair," ERBB signaling, PI3K/AKT signaling, WNT signaling, EGFR signaling, MAPK pathway, and "chromatin remodeling," as defined in the Signatures Database (MSigDB) (ver.7). In order to visualize the relationship of circRNAs to their parental genes in these pathways, we investigated if circRNA events were detected for each pathway member; if not, genes were excluded from further analysis. For the selected genes in the respective pathways, gene expression counts and circRNA counts were tabulated in two separate matrices. These matrices were then individually processed for box plot using ggplot2. To further describe the relationship of gene vs. circRNA expression, we calculated Pearson correlation values between the expression levels of the parental gene to the expression level of the circular RNAs (after normalizing the read counts for the circRNAs by library size). In order to assess potential correlations of circRNA expression to the overall pathway activity, we computed pathway signature scores (using geometric mean over the individual gene expression values, adding a pseudo-count of the lowest expression value in the set). These pathway signature scores were then correlated to the expression of the individual circRNAs in the pathway (Log_2 CPM with initial pseudo-count one) and to the relative expression (as used in the differential expression analysis).

microRNA Binding Site Prediction

To identify microRNA (miRNA) binding sites within the predicted circRNAs, we first downloaded the predicted target sites of conserved miRNA families from TargetScan (release 7.2) (31). Of the 122,607 total target sites in the dataset, there were only 192 sites with length >8 bp and, hence, only sites of length 7/8 bp (99.84% of total set) were taken forward for further analysis. This filtered target dataset was then assessed for coordinate level overlap within the circRNA coordinates using bedtools (ver. 2.27.1).

Comparison to Other Datasets

The full set of circRNAs (supported by at least two reads in at least two different samples) from the study of Okholm et al. was kindly provided by the authors. CircRNAs detected in urine by exome capture were downloaded from the supplement from Vo et al. (32) If necessary, circle coordinates were lifted over using a python script based on the python package pyliftover (version 0.4, https://pypi.org/project/pyliftover/) and slightly different coordinate notions (as start coordinate given by either 0, or +1) have been unified and entries mapped using an in-house perl script. In order to compare if the differentially expressed circRNAs identified show the same behavior between grades in the Okholm et al. data, we computed their relative frequency (occurrence in a grade grouping divided by the fraction of that

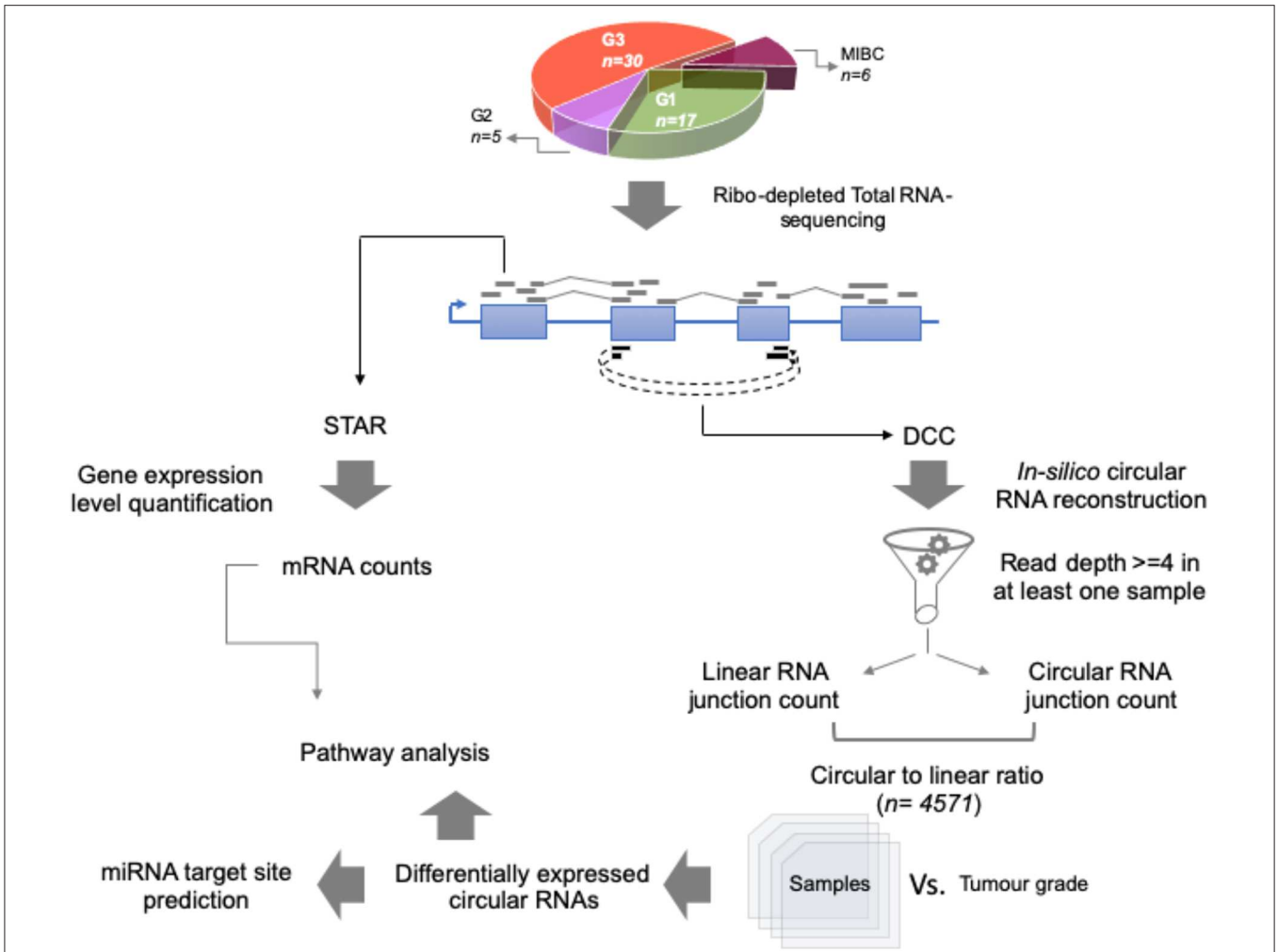

FIGURE 1 | Work flow for identification of circular RNA candidates in context of non-muscle-invasive bladder cancer subtypes. Using 58 patient tumor tissue samples across G1, G2, and G3 tumor grades for NMIBC and additionally 6 MIBC samples, total RNA-sequencing was performed using Illumina platform. Coordinate boundaries for circular RNA candidates were *in-silico* reconstructed using DCC algorithm. For each circular RNA candidate, simultaneous evidence for back-spicing and linear splicing (circular RNA count and linear RNA count, respectively) were quantified which were then used to calculate the circular-to-linear ratio for each candidate. These ratios were then subjected to differential expression statistics using the limma BioConductor package. The differentially expressed circular RNAs across the tumor grade subtypes were then used for pathway analysis based on the host gene name. Further the circular RNA sequence body was checked for harboring potential miRNA target sites using TargetScan database.

grade in the dataset) in low-grade and high-grade samples from Okholm et al. (removing the 7 papillary urothelial neoplasm samples in the set). We computed the same frequencies in our dataset by combining G1 and G2 into a "low/intermediate grade" set, and keeping G3 as high grade. For both sets, circRNAs with occurrence in at least three samples were selected for analysis. We correlated these relative frequencies using Pearson correlation.

RESULTS

Clinical Phenotype of NMIBC Patient Samples

Our study cohort includes 52 NMIBC samples (grade1/G1 $n =$ 17, grade 2/G2 $n = 5$, and grade 3/G3 $n = 30$) and 6 MIBC samples (all G3). The median age of the patients was 71 years and the male:female ratio was 6:1. There was no statistically significant difference in age distribution between the genders (Mann-Whitney p-value 0.26).

Identification of Circular RNAs

For the total RNA sequencing of all 58 tumor samples, an average of 48 million reads per sample was obtained. To comprehensively identify circular RNA candidates and query the transcriptome status we used two strategies—parallel evaluation of the linear RNA and the circular RNA landscapes (**Figure 1**).

For the circular RNA landscape, two different algorithms, CircExplorer2 and DCC, were used. While individually the two algorithms detected 4,361 and 4,571 candidates, respectively, there was significant overlap in the candidates identified

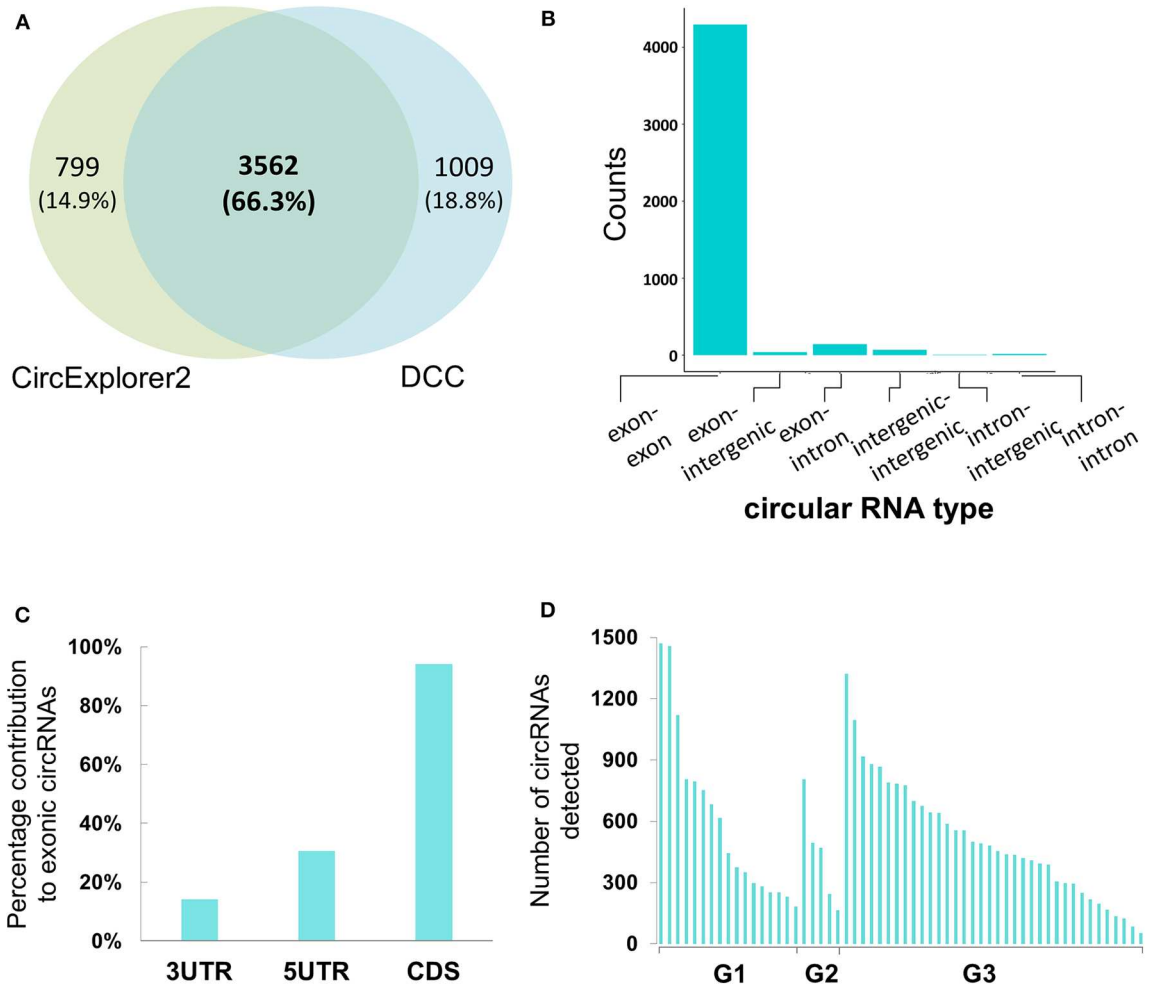

FIGURE 2 | Genomic characterization of potential circular RNAs in Bladder cancer samples **(A)** Co-ordinate level overlap between circular RNA candidates detected by CircExplorer2 and DCC, respectively. **(B)** Types of genomic origin for circular RNA candidates. X-axis is showing the genomic origin pair across the back-splicing junction. Y-axis is the count of circular RNAs obtained from that pair type. **(C)** Within mRNA positional bias for circular RNA candidates. X-axis represents the three classes of space within mature mRNA body. Y-axis is the percentage contribution of each space type to exonic circular RNA candidates. **(D)** Tumor-grade wise distribution of circular RNA candidates. X-axis represents the 58 tumor samples categorized by the tumor grades of G1, G2, and G3, respectively. Y-axis represents the number of circular RNAs detected with more than 4 back spliced reads in the tumor sample.

(**Figure 2A**). In addition to the junction count for back-splicing events, the output from the DCC algorithm also provided the paired count for the linear events which allowed comparative evaluation; hence, for downstream analyses, results from the DCC were carried forward.

Genomic Characterization of Circular RNAs

When reconstructing the boundaries of the circRNA candidates, the DCC algorithm annotates them on the basis of their genomic context (exonic, intronic, and/or intergenic); amongst all the circRNAs thus annotated, the exonic circRNAs were predominant (∼94%) (**Figure 2B**). We then assessed positional bias (within the mRNA body) of the exons involved in circRNA formation. On further resolution of exonic circRNAs over the mature RNA sections of either the untranslated region (UTR; 5′ or 3′) and coding sequence (CDS), 94.1% of the exonic circRNAs were found to be within or overlapping the CDS; the values for those within or overlapping 5′ and 3′ UTR were 30.5 and 14.1%, respectively (**Figure 2C**). In addition to these, we looked at the tumor grades for presence of more number of circular RNA candidates in one grade vs. the other. We find that the median number of circular RNAs detected when seen grade-wise are 444, 471, and 468.5 for G1, G2, and G3 respectively (**Figure 2D**). Hence there didn't appear to be any strong bias in the distribution of circRNAs across the tumor grades.

Gene Expression Differences Delineating NMIBC Tumor Grades

Gene level counts estimated from the total RNA-sequencing of the 58 tumor samples were used to identify genes with differentially expressed patterns between tumor grades. A total of 1,071 genes were identified at *p*-value threshold of 0.05 (after multiple-testing correction). Functional annotation of the gene

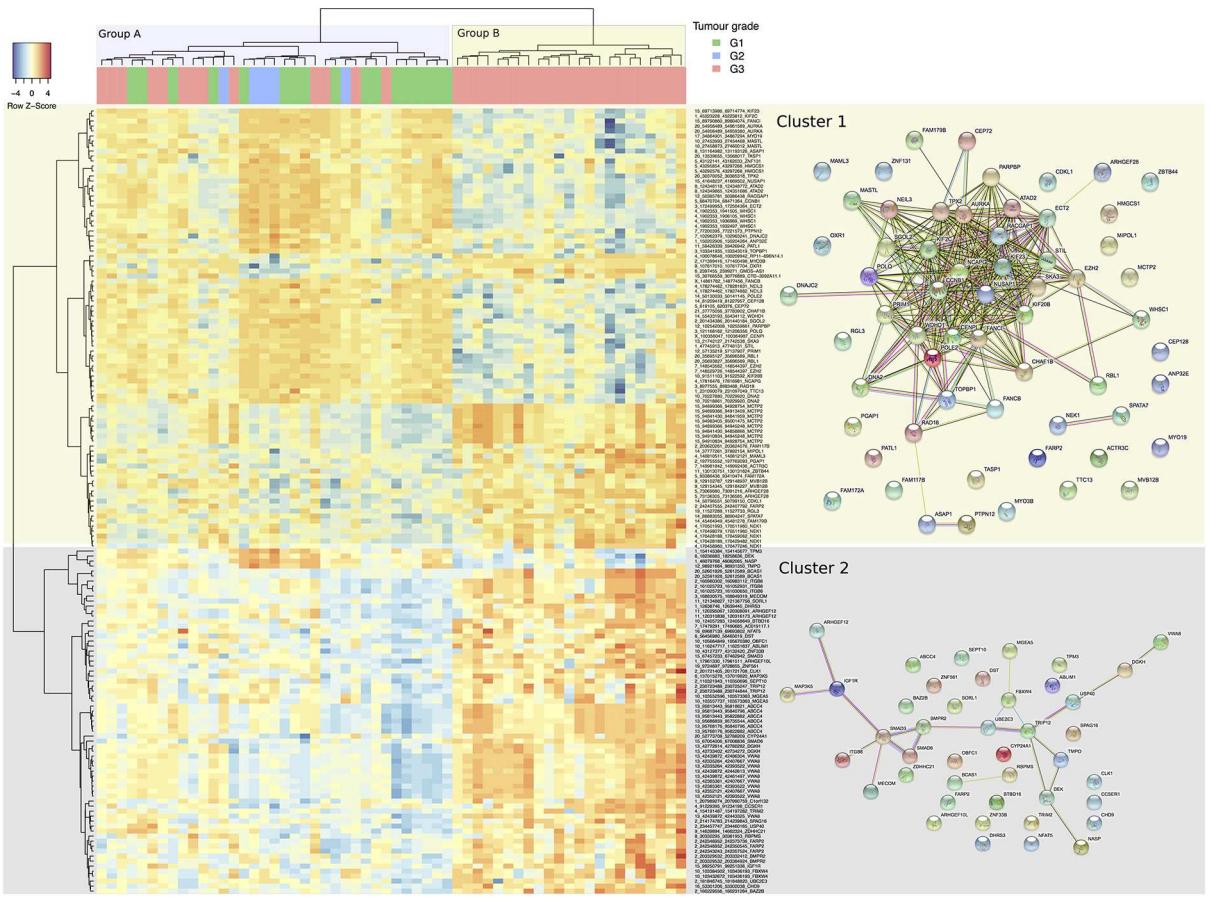

FIGURE 3 | Heatmap representation of hierarchical clustering for 157 differentially expressed circular RNAs (Y-axis) by the 58 tumor samples across G1, G2, and G3 tumor grades (X-axis). Each cell value is the circular-to-linear ratio of an individual circRNA in a given tumor sample. The two main groups of samples branching out in the cluster representation are discussed in the text are color coded in the x-axis (Group A and B), and the two main clusters of circRNAs (Cluster 1 and 2) on the y-Axis. On the right, STRING sub-networks are pictured to illustrate functional connectivity between the parental genes of the circles. Individual functional enrichments of these groups are listed in **Supplementary Table 1**.

set was performed and the pathways found to be significant were predominantly DNA-repair and cell cycle related.

Identification of Dysregulated Circular RNA Between Tumor Grades

Using the DCC algorithm at the confidence threshold of ≥4 supporting reads in at least one sample, 4,571 circRNA candidates were detected. These candidates came from 2,430 unique genes with a ratio of 1.88 circRNAs per gene. The ANOVA analysis, based on relative expression, identified 157 circRNA candidates from 107 unique host genes as having significantly different levels of expression between the tumor grades (set "differentially relative expression of circular RNAs," **Supplementary Table 1**). The parental gene list is functionally enriched in processes such as cell-cycle, DNA-repair, and cytokinesis when compared to the background of all parental genes of circRNAs detected (as reported by a GO enrichment analysis) (**Supplementary Table 1**). A heatmap based on these discriminative circRNAs is shown in (**Figure 3**), indicating their discriminative potential between tumor grades: the samples were grouped by the clustering into two main subgroups (denoted groups A and B in (**Figure 3**), separated by the initial branch, at the top of the clustering tree, on the X-axis). The first group comprises an inhomogeneous sub-cluster (A) with a mix of different grades, whereas the second main group (B) is homogenous for samples with grade 3. On the Y-axis, the circular RNAs are grouped by the clustering into two main clusters (Cluster 1 and 2). Both clusters exhibit sub-clusters with distinct expression patterns. In Cluster 1, for example, there are two sub-clusters with low expression of circRNAs predominant in the Group B samples, but rather upregulated in most of the Group A ones (mostly in the upper area of the heatmap). The parental genes of these circRNAs are enriched with functional processes relating to DNA replication, cell-cycle, and DNA repair (**Supplementary Table 1**), and reveal a highly connected module in terms of functional interactions (right panel of STRING interactions in **Figure 3**). This pattern is inverted in Cluster 2, for which Group B shows mostly high expression. However, the parental genes of circRNAs in this cluster do not exhibit a large amount of functional connectivity.

Gene Ontology enrichment (albeit not significant after correction for multiple testing) for this cluster suggests involvement of circRNAs in regulative processes including SMAD3 and 6 (indicating a potential connection to TGF-b and BMP signaling) and Insulin-Like Growth Factor 1 Receptor, implying a potential role of these circular RNAs in facilitating progression and evasion of apoptosis (**Supplementary Table 1**), nevertheless, a functional interpretation remains difficult. Compared with a clustering based upon significantly differentially expressed genes (**Supplementary Figure 1**), the clustering appears of similar if not better discrimination between grades: as mentioned, Group B comprises Grade 3 tumors only with a distinct expression pattern of circRNAs. A separate but large fraction of Grade 3 tumors are clustered together with Grade 1 and Grade 2 samples in Group A. Grade 2 exhibits a tendency to cluster together in Group A which shows high expression of a small sub-set of circular RNAs (with parental genes TPM3, DEK, NASP, and TMPO) and which is low for most members of Group B.

We also collected circRNA candidates whose parental genes are affected by NMIBC grade as detected by differential expression analysis on the gene level: the set of all genes with any circRNA detected (n = 2,430) was overlapped with the set of differentially expressed genes (n = 1,071; **Supplementary Figure 1**). This resulted in an intersection of 143 host genes that are differentially expressed between tumor grades and exhibit circularization (set "gene differentially expressed at mRNA level," **Supplementary Table 1**). Finally, genes where the circRNAs are differentially expressed with little influence of the host gene expression dynamic between the different grades were identified by subtracting the "circles in differentially expressed genes ($n = 107$)" set from the "differentially relative expression of circular RNAs" set (n = 143): 24 such circRNAs were identified (**Supplementary Table 1**). These 24 circRNAs did not exhibit functional enrichment.

Pathway Analysis

Visual inspection of the expression levels of linear RNA and circRNA (gene level summarized) for genes with circRNA in bladder cancer relevant pathways (**Supplementary Figure 2**), indicates non-regular patterns of circRNA expression which do not follow obvious trends in relation to the linear form. Whereas, for the linear RNA the spread of expression level per gene tended to be more tightly bound, the circRNA spread was much more variable across genes, samples and tumor grades. In concordance, most circRNA expression levels show only poor correlation to the expression of the parental gene (**Supplementary Table 3**). In general, the expression of individual circRNAs within these pathways does not correlate with the overall pathway activity (**Supplementary Table 2**). This is also the case when correlating the relative circular expression with the pathway activity. However, more significantly correlated instances can be detected in the latter analysis (at a P-value cut-off at 0.05: 66 for the relative vs. 174 for the overall expression, **Supplementary Table 2**), for example, a circRNA originating from FANCI, a gene involved in DNA repair, shows a strong negative correlation with the DNA repair process ($R = -0.85$, $P < 2.2^{-16}$).

Frequency of MicroRNA (miRNA) Binding Sites Within circRNAs

Using the TargetScan dataset of predicted miRNA target sites, a total of 132 circRNAs (hosted by 99 unique genes) were found to harbor target sites for 141 miRNAs. Amongst the host genes whose circRNAs harbor miRNA target sites, 4 were differentially expressed (both at the mRNA and at circRNA level) between the tumor grade subtypes: *CDKL1*, *HP1BP3*, *MVB12B,* and *TMPO*. The circRNAs from these 4 host genes harbor target sites for 11 different miRNAs: miR-7-5p, miR-30-5p, miR-31-5p, miR-96-5p/1271-5p, miR-139-5p, miR-181-5p, miR-182-5p, miR-433-3p, miR-489-3p, miR-493-5p, and miR-543. The 5 most recurrent miRNAs with target sites within circRNAs are the miR-15/107 family, miR-101-3p, miR-204-5p/211-5p, and miR-203a-3p. Many of these miRNAs have been reported to be implicated in tumor biology and/or be predictive of response to drug treatment (33–38). The results are summarized in (**Supplementary Table 1**).

Comparison to Other Datasets

The comparison between our dataset and that of Okholm et al. identified 3,361 overlapping circRNAs [77% from this study, 22% of the 15,223 instances in the data of Okholm et al. (21)] (all instances listed in **Supplementary Table 4**). For the circRNAs significantly differentially expressed between grades, 108 could be found in both datasets (69%). To each of these 108 circRNAs we assigned their relative frequency to occur in the set of low/medium and high-grade tumors, respectively, to describe their tendency to be grade specific. We compared these between the two datasets and found a strong correlation ($R = 0.47$, $p = 2.3e{-}07$; **Supplementary Figure 3**), indicating that their grade-specific behavior can be detected independent of the cohort. Notably, the identified grade-stratifying candidate circRNAs do not include the two proposed progression biomarkers identified by Okholm et al., circHIPK3 and circCDYL.

We identified 552 of our 4,116 circRNAs (12%) in the urine dataset from 13 prostate cancer patients provided by Vo et al. (32). This urine dataset comprises 1,092 circRNAs, resulting in a 50% recall of the urinary circRNAs in at least one of our samples. Within the set of significant differentially expressed genes (n = 157), 8% (13 circRNAs) can be found in the urine dataset (**Supplementary Table 4**).

DISCUSSION

In this work we have investigated circRNAs from a cohort of NMIBC patients. The general genomic properties of the circRNAs are in keeping with earlier findings in other studies: circRNAs are mostly connecting exons, and are mostly detected within coding sequences. The total number detected varied between tumors and was lowest in grade 2 tumors (although only 5 grade 2 tumors were analyzed). However, detected number of individual circRNAs was always >100 and up to >1,000, providing a considerable dataset for further investigations. Since our dataset is well-annotated by tumor grade (G1, G2, and G3), we aimed to delineate a subset that stratified the

dataset by grade using a supervised approach. This approach selected 157 circRNAs, and cluster analysis resulted in distinct subgroups of tumors (**Figure 3**). The clustering presented here is a visualization of the results of the differential relative circular expression analysis. The resulting heatmap might slightly vary by choice of parameters and clustering algorithms; however, the result implies the existence of molecular subtypes in terms of circRNA expression, with varying tendencies of certain grades to exhibit the subtype.

Gene expression differences among NMIBCs have been reported previously, and pathways relevant to tumor biology in bladder cancer are ERBB2, PI3K-AKT, cell cycle, MAPK, and DNA-damage repair. One of the primary questions driving investigation into any form of cis-regulatory RNA is how it affects the expression level of the host gene. In this regard, we investigated the comparative expression levels of the linear and circular RNA for selected pathways. We observe that, for a given pathway, the genes (linear RNA) retain a similar trend of expression pattern between tumor grades, whereas the circRNA levels are much more variable. This could be an effect of increased variability in the circRNA count data since the majority are at low expression levels compared to linear RNA. Nonetheless, for a particular grade group, we observe a discernible shift in the expression pattern of some genes. In the ERBB2 pathway, the *ERBB2* gene is consistently the highest expressing linear gene among all the three tumor grades but, for circRNA, *SOS2* has the highest expression across the grades. Similarly, in the PI3K-AKT pathway, *ACTR3*, *SMAD2*, *STAT2,* and *ACACA* are most highly expressed in linear RNA, whereas for circRNA, *MAPK8* appears to have higher expression. These observations indicate that within the same pathway there are likely different regulatory processes acting on linear vs. circular RNA. This is also indicated by the lack of correlation between the expression levels of circRNAs to their parental genes in the investigated pathways.

We also found that the activation of a pathway (as measured by gene signature score) is mostly independent of the circRNAs within the pathway, but less so when looking at relative expression. These instances, for which the relative expression either positively or negatively correlates with the pathway expression, might be under certain biological constraints. In the case of a negative correlation, the cancer cell seems to avoid upregulation of circRNA and the observation could be explained by a steady level of circRNAs with increasing pathway activity. With a positive correlation there may be a cis-acting role on the pathways, since their relative expression increases with pathway activity but is not bound to the expression of the parental gene. The way by which these circRNAs potentially interact with pathways of interest cannot be directly deduced from this analysis. However, systematic correlation studies may help to identify candidates for functional follow up screens.

Interestingly, four predicted miRNA sponges could be found differentially expressed. We found cancer-related pathway-specific genes being targets of the top recurrent miRNAs (targeting circRNA). The miR-15/107 family is found to have target sites within circRNAs from host genes *CDC42*, *MGEA5*, *CHPT1*, *GPATCH2L*, *TSEN2*, *RPL14*, *PLD1*, *TFRC*, *PDLIM5,* and *PTK2*. The genes *CDC42*, *PLD1,* and *PTK2* are involved in the EGFR signaling pathway; the miR-15/107 family is reported to have tumor suppressor properties (34, 39), and the circRNAs from the above genes for EGFR signaling pathway can act as "sponges" to exert oncogenic effect in the context of NMIBC. Similarly, miR-204-5p/211-5p has targets within *CDC42*, *KHDRBS1*, *ASH1L*, *MDM2*, *GPATCH2L*, *WSB1*, *ZNF638,* and *AGTPBP1* genes. Along with *CDC42*, *MDM2* is involved in DNA damage responses. Additionally, the duo of miR-204-5p/211-5p has recently been reported to be involved in resistance to BRAF inhibition (40). Hence, circRNAs harboring target sites for miR-204-5p/211-5p can have important implications for tumor treatment and progression (41, 42).

The number of samples in our study is still small, especially for Grade 1 and Grade 2 samples and the reported list of differentially expressed circRNAs provides a list of interesting candidates that can be tested in future studies. However, the existence of another bladder cancer cohort suitable for circRNA detection by Okholm et al. gave us the opportunity to compare two different cohorts and to test our findings in their data. The grade-specific relationships of circRNAs could be identified in the dataset of Okholm et al. (21). This indicates a certain amount of transferability between independent cohorts, despite different computational pipelines, and provides some mutual validation of the two cohorts. The urinary circRNA in the dataset from prostate cancer patients provided by Vo et al. would be expected to comprise both prostate cancer specific circRNAs and circular RNAs from normal bladder tissue, and was therefore suitable to investigate whether circRNAs detected in our study can be detected in urine. Indeed, we found 50% of the circular RNAs in the urine set in at least one of our bladder tumor samples, and 12% of the bladder tumor circRNAs from this study exist in the urine dataset. It is therefore likely that a high proportion of circRNAs expressed by bladder tumors will be detectible in urine, notwithstanding the circRNAs differentiating between grades are slightly under-represented with 8% recall. The latter observation might indicate that the discriminative set comprises instances that are bladder cancer specific; however, given the relatively small size of this dataset, the significance of this finding remains unclear. Nevertheless, these observations indicate a need to further investigate the potential of urinary circRNAs as diagnostic and/or prognostic biomarkers.

CONCLUSION

The present study provides a step in the comprehensive evaluation of circRNAs in the context of bladder cancer. Intriguingly, despite their potential function as microRNA sponges, circRNAs are potentially affecting host mRNA levels at the transcriptional stage, as compared to post-transcriptional control by miRNAs. We have also identified circRNA candidates worthy of further functional investigation, and comprising potential miRNA sponges and circRNAs correlated to pathway expression. Our analysis indicates circRNA differences between bladder cancer grades, and their relative expression levels may provide an additional modality for patient risk stratification. Furthermore, since circRNAs have a longer half-life in the

extracellular milieu than other RNAs and are detectable in urine, they may be useful non-invasive biomarkers for bladder cancer diagnosis and risk stratification.

DATA AVAILABILITY STATEMENT

RNA sequencing data for the bladder cancer patients have been submitted at the European Genome-Phenome Archive (EGA) (https://ega-archive.org/) under accession code: EGAS00001004358.

AUTHOR CONTRIBUTIONS

AG, RA, and RB conceived and designed the study. NG and DW provided technical and material support. BA, MZ, KC, NJ, and RB have been involved in acquisition of funding, samples, and data. AG and RA analyzed the data, AG, RA, DW, and RB wrote and revised the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00812/full#supplementary-material

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Conflict of Interest: RB has contributed to advisory boards for Olympus Medical Systems and Janssen. NJ has contributed to advisory boards for Merck USA and Pierre Fabre.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with several of the authors RA, AG, DW, NG, BA, MZ, KC, NJ, and RB.

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