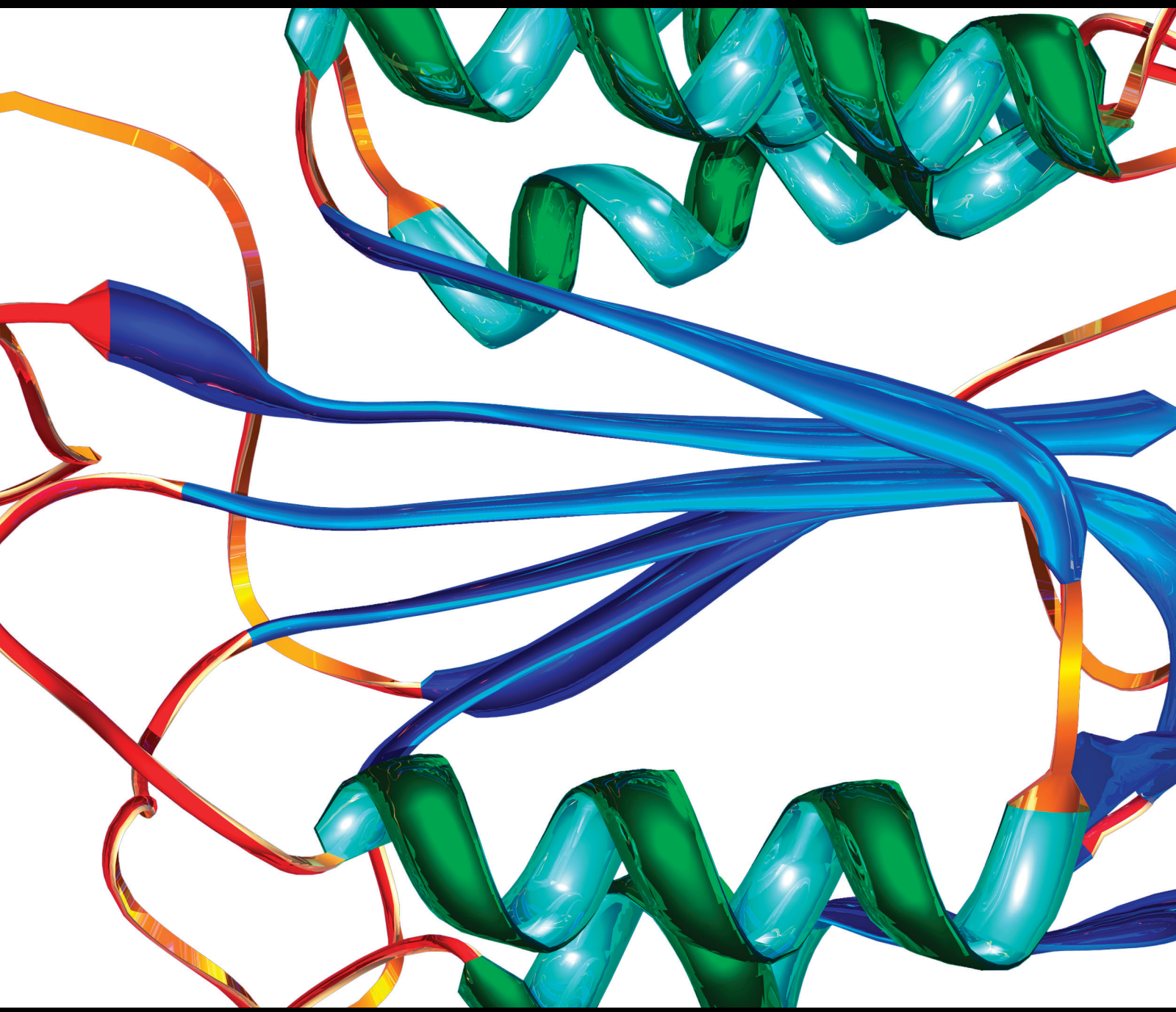
Disease Markers

Diagnostic and Prognostic Markers in Bladder Cancer

Guest Editors: Ja Hyeon Ku, Wun-Jae Kim, Seth P. Lerner, Felix Chun, and Luis Alex Kluth



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Editorial

Diagnostic and Prognostic Markers in Bladder Cancer

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Bladder cancer has the highest recurrence rate of any solid tumor. Due to the need for life-long surveillance, the per-patient cost of managing bladder cancer is among the highest for any cancer. There has been growing activity in the development of novel markers for bladder cancer detection; the ultimate goal of these novel markers would be to replace and/or complement cystoscopy as a sensitive and noninvasive surveillance tool. Besides, with recent advances in techniques, an extremely large number of new prognostic markers have been identified. New biomarkers that are predictive of outcomes would help clinicians provide the risk-stratification of patients and serve as prognostic indicators for individual patients. Continuing identification of new markers in bladder cancer will enhance future diagnostic and therapeutic approaches to bladder cancer.

We invited investigators to contribute articles that will stimulate continuing efforts to leverage biomarkers to improve diagnostic accuracy, discover the molecular pathophysiology underlying bladder cancer, develop strategies to treat these conditions, and evaluate the prognosis. The purpose of this special issue is to give a comprehensive overview of the current state of knowledge regarding diagnostic and prognostic markers in bladder cancer.

Several studies have indicated that steroid hormones and their receptor signals, especially androgens/estrogens and androgen/estrogen receptors, in bladder cancer, have critical roles in tumorigenesis and tumor progression. In one review, H. Ide and H. Miyamoto summarized that steroid hormone receptors and related signals can serve as biomarkers of urothelial tumors, especially their prognosticators.

In "Association of Cytokeratin and Vimentin Protein in the Genesis of Transitional Cell Carcinoma of Urinary Bladder Patients," A. H. Rahmani et al. performed immunohistochemistry and investigated cytokeratin and vimentin in urothelial cancer cases and inflammatory lesions. They concluded that both markers cytokeratin and vimentin will be helpful markers in the early diagnosis of urothelial carcinoma.

M. S. Wettstein et al. evaluated CD73 expression immunohistochemically in 174 patients with a primary urothelial carcinoma. They found that high CD73 expression was associated with favorable clinicopathological features such as lower stage, lower grade, less adjacent carcinoma in situ, and lower Ki-67 proliferation index as well as with better outcome.

H. Yang et al. investigated the correlation between the urine soluble Fas (sFas) and vascular endothelial growth factor (VEGF) in patients with urothelial bladder carcinoma. They reported that the urinary sFas levels and the VEGF expression were correlated significantly and they might play important roles in the occurrence and progression of urothelial bladder cancer.

In the case-control study, S. Choi et al. assessed urinary apurinic/apyrimidine endonuclease 1/redox factor-1 (APE1/Ref-1) by enzyme-linked immunosorbent assay (ELISA) in 169 bladder cancer patients and 108 nonbladder cancer controls. APE1/Ref-1 levels were significantly elevated in bladder

cancer patients compared to those in controls and were correlated with tumor grade and stage. These findings suggest that urinary APE1/Ref-1 levels would be clinically applicable for diagnosis of bladder cancer.

Plasmacytoid urothelial carcinoma is a rare and aggressive variant form and little is known about HER2 protein expression and gene alterations. B. Kim et al. demonstrated that HER2 protein overexpression was frequently found and suggested that HER2 may be a potential therapeutic target for this variant form.

The available omics data may allow us to elucidate the mechanisms behind bladder carcinogenesis. According to the omics data on human cells, C.-W. Li and B.-S. Chen constructed an integrated genetic and epigenetic network system (IGEN) based on three coupling regression models. They demonstrate that an accurate genome-wide IGEN would allow us not only to elucidate bladder carcinogenesis mechanisms, but also to improve drug safety and efficacy in the treatment of bladder cancer.

The review by Y. Miyata and H. Sakai demonstrated the various histological and molecular markers for recurrence after intravesical therapy in patients with nonmuscle-invasive bladder cancer. Because intravesical therapy is usually performed after transurethral resection, they discussed the results obtained from tissue samples regarding the various cancer-related molecules, immunity-related factors, and gene polymorphism.

M. Nagata et al. reported the review article discussing the application of molecular predictive biomarkers in patients with advanced muscle-invasive bladder cancer as well as in postcystectomy patients. They also discuss the current findings of liquid biopsy in patients with advanced bladder cancer.

In this special issue, H. S. Kim and J. H. Ku reviewed the clinical studies dealing with systemic inflammatory response (SIR) related biomarkers, with a special focus on neutrophil-to-lymphocyte ratio (NLR). Elevated NLR has shown a significant association with adverse outcomes in patients with carcinoma of upper urinary tract as well as bladder. Since NLR may be an inexpensive and reproducible measurement, it might become a promising tool in the management of urothelial carcinoma.

In one article of this issue, X. Gan et al. conducted a systematic review and meta-analysis and identified an important link between downregulated p16 expression and poor prognosis in patients with bladder cancer. They concluded that p16 plays an essential role in deterioration of bladder cancer and could serve as a biomarker for patients' prognosis.

Taken together, we believe that this special issue gives a comprehensive overview of the contemporary area of diagnostic and prognostic markers in bladder cancer. We hope this special issue is useful to researchers and clinicians to research of bladder cancer.

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We would like to thank the editorial office for creating this special issue, the authors who have contributed their time, and all the reviewers for critical review of the manuscripts.

Ja Hyeon Ku
Wun-Jae Kim
Seth P. Lerner
Felix Chun
Luis Alex Kluth

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Review Article

Prognostic and Clinicopathological Significance of Downregulated p16 Expression in Patients with Bladder Cancer: A Systematic Review and Meta-Analysis

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p16, encoded by the *CDKN2A* gene, is a tumor suppressor that has been widely studied in cancer research. However, the relationship of p16 with prognostic and clinicopathological parameters in patients with bladder cancer remains unclear. Data inclusion criteria were articles reporting on the relationship between p16 expression and the prognosis or clinicopathology in patients with bladder cancer. Meta-analyses were performed with Stata software. Hazard ratios (HRs) or odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to evaluate the relative risks. The source of heterogeneity was analyzed by subgroup analysis. A total of 37 studies with 2246 cases were included and analyzed. The results identified an important link between downregulated p16 expression and poor prognosis in patients with bladder cancer in terms of recurrence-free survival (RFS), overall survival (OS), progression-free survival (PFS), and some clinicopathological parameters including clinical staging, pathological degree, and lymph node metastasis. Subgroup analysis also showed that low p16 expression could function as a warning sign for RFS and PFS in patients with early-stage (Ta–T1) bladder cancer. In conclusion, p16 might play an essential role in the deterioration of bladder cancer and could serve as a biomarker for the prediction for patients' progression and prognosis.

1. Introduction

Bladder cancer is the most frequent malignancy of the urinary tract and the ninth most common cancer worldwide [1]. About 95% of bladder cancers are histologically transitional cell carcinoma, with rare cases of squamous cell carcinoma and adenocarcinoma. However, the pathogenesis of bladder cancer is still unclear, and its occurrence and development appear to be affected by multiple genes [2]. Serrano et al. first cloned the cDNA of the gene encoding the tumor suppressor protein p16 (CDNK2A) in 1993; since then it has been widely studied in the field of cancer research [3]. Previous studies have reported ubiquitous downregulation of p16 gene expression in bladder cancer, as a result of various alterations including complete deletion, point mutation, or promoter methylation [4–6]. Furthermore, p16 could compete with cyclin D1 for binding to Cyclin Dependent Kinase (CDK) 4/6, thus blocking the phosphorylation of retinoblastoma (Rb) protein and inhibiting release of the transcription factor E2F, preventing cell conversion from G_1 phase to S phase, and eventually suppressing cell proliferation. These results suggest that abnormal expression of the p16 gene in cells might be associated with tumorigenesis [6, 7].

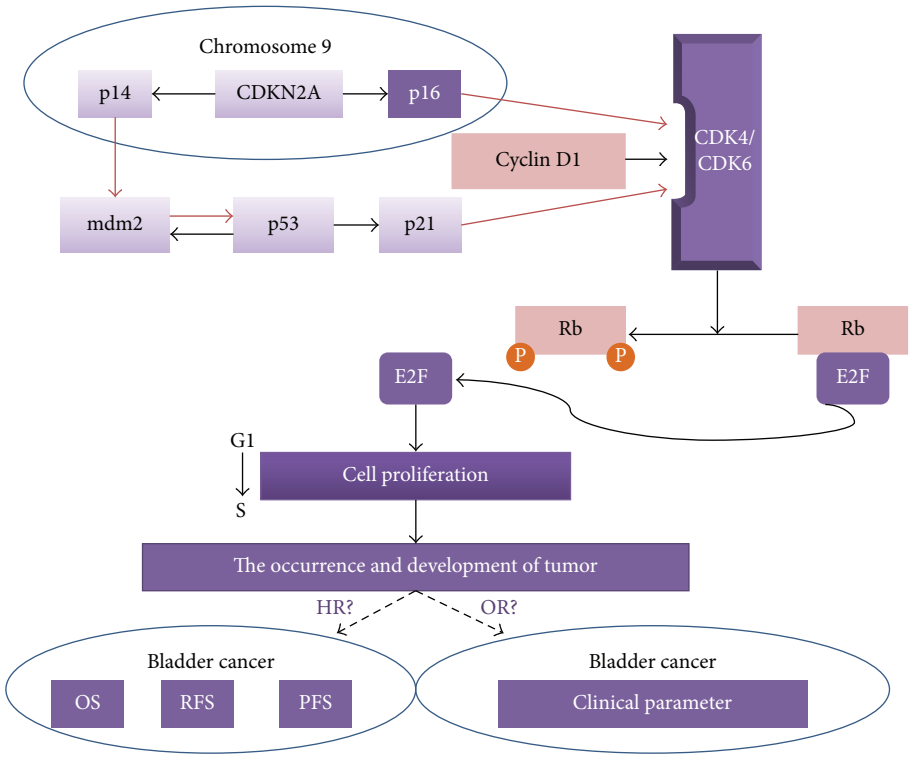


Figure 1: Main molecular pathways of bladder cancer (adapted from Mitra et al. [7]).

Numerous studies to date have explored the clinicopathological and prognostic significance of p16 in patients with bladder cancer. However, as a result of differences in sample sizes, accuracies of the statistical data, study populations, and interventions, the results remain inconclusive, and evidence-based confirmation by large-scale clinical trials is still lacking. We therefore conducted an in-depth systematic review and meta-analysis to investigate the correlation between abnormal expression of p16 and clinicopathological features, as well as prognosis in patients with bladder cancer. The specific mechanisms are shown in Figure 1.

2. Materials and Methods

2.1. Literature Search. The terms and combinations including "Cyclin Dependent Kinase Inhibitor p16," "CDKN2A Protein," "p16INK4A Protein," "MTS1 Protein," "Cyclin Dependent Kinase Inhibitor 2A," "Multiple Tumor Suppressor 1," "Cdk4 Associated Protein p16," "TP16," and "urinary bladder neoplasms," "bladder tumors," "bladder cancers," "bladder carcinomas," and "prognos*," "surviv*," "follow-up," "mortality," "predict," "course," "outcome," and "clinicopathological" were used to search the following domestic and international databases: PubMed, Wiley Online Library, Embase, Cochrane Central Register of Controlled Trials, Science Direct, EBSCO, Google Scholar, Ovid, LILACS, China National Knowledge Infrastructure (CNKI), China Biology Medicine disc (CBMdisc), CQVIP, and Wan Fang, with unified retrieval rules such as Boolean logic. The obtained search results were then analyzed, evaluated, reviewed, and manually screened to determine their relevance.

2.2. Inclusion and Exclusion Criteria. Inclusion criteria were as follows: (1) patients diagnosed with bladder cancer; (2) immunohistochemical (IHC) detection of p16 expression levels in the tissues; (3) relationships between abnormal expression of p16 and prognostic indicators such as recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS) or associations between p16 and clinicopathological features that were evaluated; (4) hazard ratio (HR), odds ratio (OR), relative risk (RR), and 95% confidence intervals (CI) that could be obtained directly from the full article or indirectly calculated with relevant software based on the data provided in the graphics and tables; (5) only the newest studies or the ones with higher quality were retained if the data were repeated in different studies; and (6) studies in English or Chinese.

Exclusion criteria were as follows: (1) cell or animal studies, case reports, letters, reviews, and meta-analyses; (2) articles with similar content or using the same data or those with small sample sizes ($n \leq 10$) and those with no directly or indirectly extractable HR, OR, and 95% CI data; and (3) articles that could not be understood because of language barriers.

2.3. Data Extraction. Two independent investigators (Xiaoning Gan and Rongquan He) reviewed the articles that met the criteria and extracted data on author, year of publication, nationality, sample size, patient age, detection method of p16, antibody source and dilution, clinical stage, pathological degree, other costudied prognosis-associated genes, cut-off value, outcome, and extraction method of the study subjects. Discrepancies between the two independent investigators in

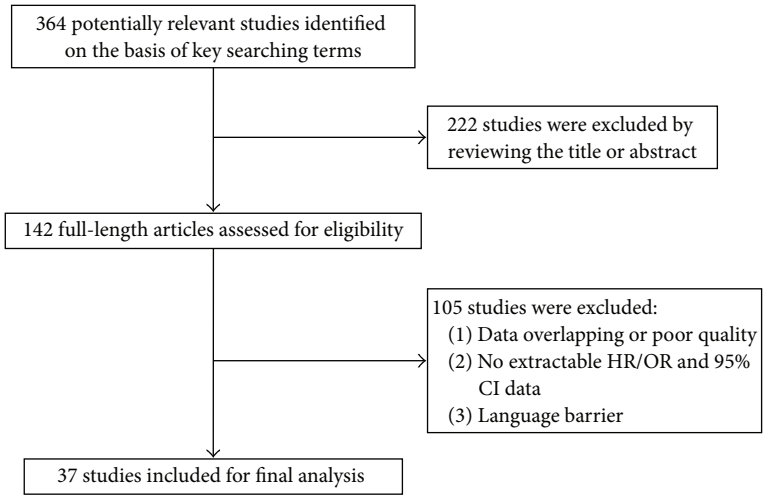


Figure 2: Flow diagram of studies selection procedure.

terms of data extraction were resolved by discussion among all the authors.

2.4. Statistical Analysis. Effects of p16 on the related prognostic indexes were detected by merging the HRs and 95% CI of the included literatures, which were evaluated through the Forest plot and related parameters after the merging. The HRs and 95% CI values mainly came from direct extraction of the original text or survival curve through extraction and calculation by software.

The relationships between p16 and the clinicopathological parameters were derived from the binary variable data extracted from the original articles. ORs and 95% CI values came from the binary variable data calculated by Stata software. The data were then combined, and their statistical significance was evaluated by Forest plot and related parameters, to clarify the relationship between p16 low-expression and clinicopathological parameters.

Heterogeneity was measured by Q statistics as follows: no heterogeneity: $0 < I^2 < 25\%$; low heterogeneity: $25\% \leq I^2 < 50\%$; moderate heterogeneity: $50\% \leq I^2 < 75\%$; high heterogeneity: $75\% \leq I^2 \leq 100\%$. If $I^2 < 50\%$ and $P > 0.10$, a fixed-effect model would be used in combination with HRs, ORs, and 95% CI; if $I^2 \geq 50\%$ and $P \leq 0.10$, then a random-effect model would be selected. Heterogeneity analysis was performed to assess the accuracy of the data, and subgroup and sensitivity analyses were carried out based on professional knowledge.

Publication bias was detected by Begg's funnel plot and Egger's test with Stata software. A two-sided P value < 0.05 was considered to indicate statistical significance. Statistical analyses were carried out with StataSE 12.0, Engauge, Photoshop CS5, and Microsoft Office 2007.

3. Results

3.1. Eligible Studies. A total of 364 articles were identified from the databases, including 190 English and 174 Chinese articles, 222 of which were excluded because of discrepancies between the study theme and their abstracts. The full text of the remaining 142 articles was then reviewed for their fit with the current study, after which a further 105 articles were excluded because they met one or more of the exclusion criteria, such as the cell or animal studies, reviews, and letters and studies with identical data and no extractable HR, OR, and 95% CI data from the full text or language barrier. The remaining 37 articles [4, 5, 8–42] with 2246 cases were included in our study and consisted of 21 English [4, 5, 8–19, 21–25, 41, 42] and 16 Chinese [20, 26–40] articles. The screening process was demonstrated in Figure 2.

The basic features of the included studies were presented in Table 1. Among the 37 articles, 26 studies [4, 5, 8–21, 28–34, 39, 41, 42] investigated the relationship between low expression of p16 and prognostic parameters in bladder cancer patients (RFS, OS, PFS, and DSS/CSS), and 30 studies [4, 10–13, 15–19, 21–40] assessed the association between p16 and clinicopathological factors in patients with bladder cancer.

3.2. Relationship between Downregulated p16 Expression and RFS in Patients with Bladder Cancer. A total of 17 studies with 1032 subjects were included in the final analysis of RFS [4, 5, 8, 10, 13–15, 19, 21, 28–34, 39]. Low expression of p16 was related to poor RFS in patients with bladder cancer (HR = 1.63, 95% CI = 1.36~1.94, and $P < 0.001$), with low observed heterogeneity ($I^2 = 42.6\%$, $P = 0.029$) (Figure 3(a)).

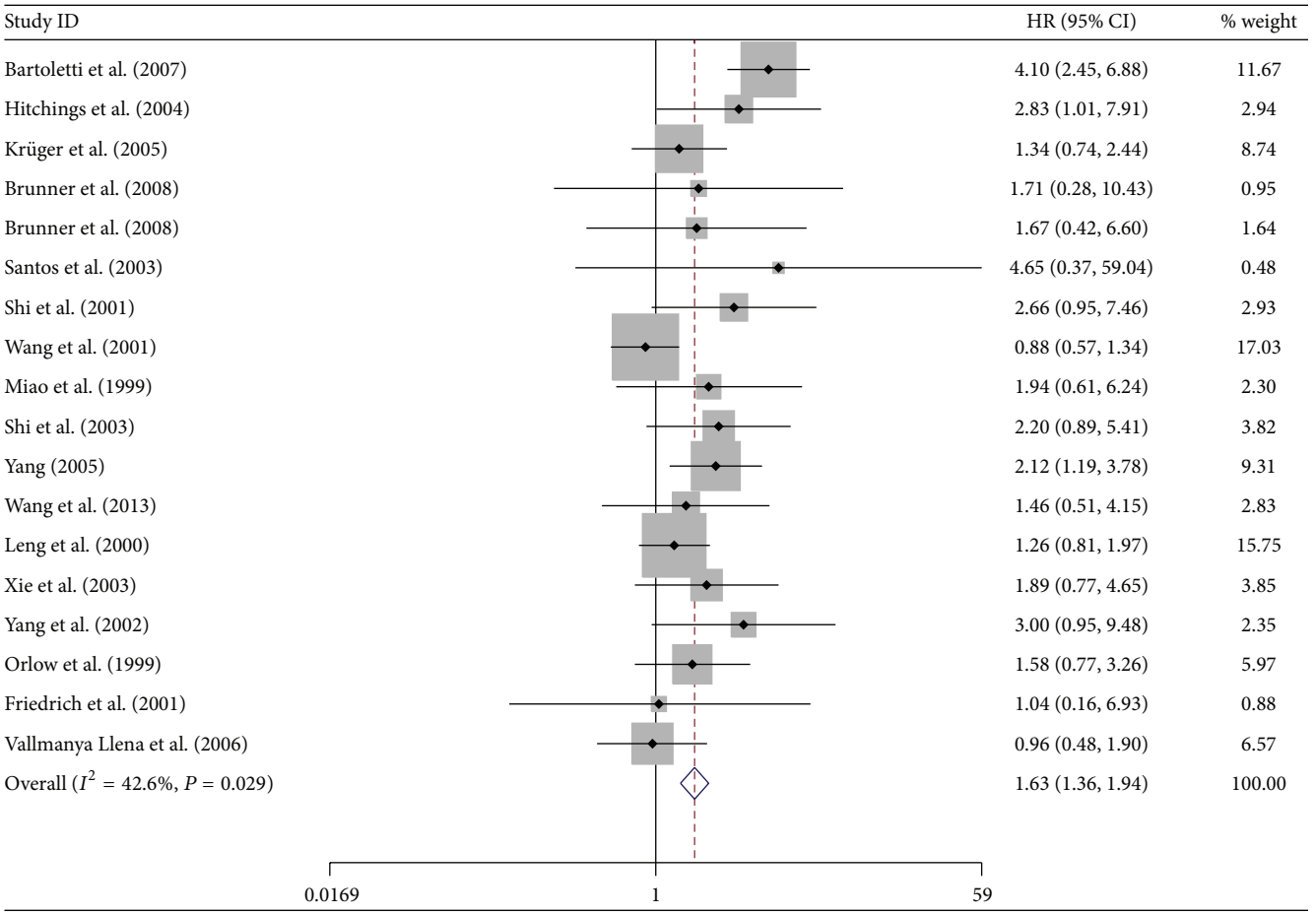
Cumulative meta-analysis based on year of publication and sample size demonstrated that the results tended to stabilize with increasing sample size, but there was no obvious relationship between the results and year of publication.

Based on sensitivity analysis, the study by Yang et al. [13] was initially excluded because of a large difference in HR compared with the overall average, which was attributed to the selection of a different calculation method in the original article. Binary variable data were extracted and the HR and 95% CI were therefore recalculated with Stata software.

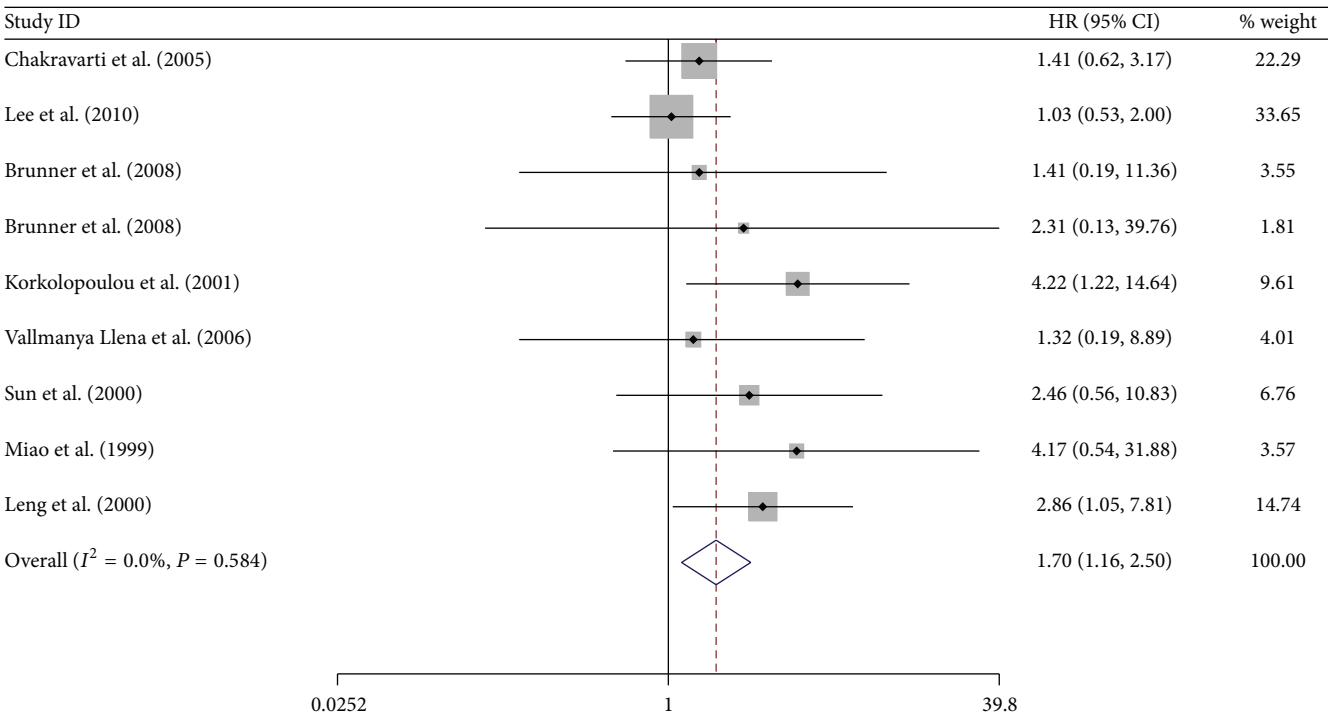
Table 1: Main features of all studies included in the meta-analysis.

Author	Year	Nation	No.[b] (M/F)	Age	Stage	Grade	Cut-off value	Outcome	Data extraction	Other costudied genes	Antibody source (dilution)	Detection method of p16
Orlow et al. [4]	1999	Canada	120	NR	Ta–T1	G1–G3	Score = 3	RFS/CP	Reported	P14	Vector (1 : 500)	Immunohistochemistry
Bartoletti et al. [8]	2007	Italy	56 (50/6)	70.1 (45–89)	Ta–T1	G1-G2	10%	RFS	Reported	9p21	Bio-Optica (1 : 25)	Immunohistochemistry
Chakravarti et al. [9]	2005	USA	50 (36/14)	NR	T2–T4	High	20%	OS/FFS/DSS	Reported	Erb-1, Erb-2, P53, PRB	Zymed (NR)	Immunohistochemistry
Hitchings et al. [10]	2004	UK	78	66 (24–90)	Ta–T1	G1–G3	10%	PFS/RFS/CP	Reported	P53, PRB	Novocastra (1 : 50)	Immunohistochemistry
Krüger et al. [5]	2005	Germany	73 (60/13)	68 (NR)	T1	G2-G3	10%	RFS/PFS	Reported	NR	Biocarta (1 : 50)	Immunohistochemistry
Lee et al. [11]	2010	Korea	47 (4/43)	NR	Ta–T4	Low and high	Score = 5	OS/CP	Reported	P53, PRB	DAKO (1 : 200)	Immunohistochemistry
Mhawech et al. [12]	2004	Switzerland	49 (44/5)	70.3 (52–90)	T1	Low and high	Score = 3	PFS/CP	Reported	P21	DAKO (1 : 20)	Immunohistochemistry
Yang et al. [13]	2002	China	67	NR	T1-T2	G1–G3	5%	RFS/CP	Binary variable	Cyclin D1, CCNE, p27, p21, p53	Santa Cruz (NR)	Immunohistochemistry
Brunner et al. [14]	2008	Switzerland	99	NR	Ta–T4	Low and high	1.5% or 23%	OS/RFS	Survival curve	MTS	NeoMarkers (1 : 50)	Immunohistochemistry
Friedrich et al. [15]	2001	Germany	40	NR	Ta–T1	G1–G3	5%	RFS/CP	Survival curve	LOH	Pharmingen (1 : 100)	Immunohistochemistry
Korkolopoulou et al. [16]	2001	Greece	23	72 (35–92)	T3-T4	Low and high	5%	OS/CP	Survival curve	P53	Santa Cruz (1 : 100)	Immunohistochemistry
Niehans et al. [17]	1999	USA	78	64.7 (48–82)	T1–T4	G2–G4	Score = 4	DSS/CP	Survival curve	P53, PRB, cyclin D1	Pharmingen (1 : 400)	Immunohistochemistry
Røtterud et al. [18]	2002	Norway	59	64 (42–75)	T2–T4	G2-G3	Score = 3	CSS/CP	Survival curve	p21, p27	NeoMarkers (1 : 100)	Immunohistochemistry
Vallmanya Llena et al. [19]	2006	Spain	97	NR	Ta–T1	Low and high	15%	RFS/PFS/OS/CP	Survival curve	p53, p21	DakoCytomation (NR)	Immunohistochemistry
Sun et al. [20]	2000	China	60	NR	Tis–T4	G1–G3	Score = 4	OS	Survival curve	PRb	Santa Cruz (1 : 100)	Immunohistochemistry
Santos et al. [21]	2003	Portugal	56 (40/16)	70 (43–83)	Ta–T1	G1-G2	20%	RFS/CP	Binary variable	p27, pRb, p53, Ki-67	Pharmingen (1 : 500)	Immunohistochemistry
Yin et al. [22]	2008	USA	18	NR	T1–T4	Low and high	Score = 4	CP	Binary variable	9p21	Pharmingen (1 : 250)	Immunohistochemistry
Primdahl et al. [23]	2002	Denmark	69 (55/14)	71 (42–83)	Ta–T4	G1–G4	Score = 4	CP	Binary variable	Rb, p27, p21, L-myc	NeoMarkers (1 : 50)	Immunohistochemistry
Jin et al. [24]	2006	USA	39 (25/14)	65 (42–84)	T2–T4	G1–G4	10%	CP	Binary variable	P53, pRB	NR (1 : 50)	Immunohistochemistry
Tzai et al. [25]	2004	China (Taiwan)	65 (44/21)	61.5 (41–84)	T2–T4	G2-G3	Score = 4	CP	Binary variable	P53, pRB	Santa Cruz (1 : 20)	Immunohistochemistry
Jin et al. [26]	2004	China	62 (32/30)	61 (18–80)	Tis–T4	G1–G3	OC	CP	Binary variable	Cyclin D1, PCNA	NR	Immunohistochemistry
Fu and Li [27]	2011	China	50 (39/11)	59.3 (32–81)	Tis–T4	G1–G3	10%	CP	Binary variable	E-cadherin	NR	Immunohistochemistry
Shi et al. [28]	2001	China	62 (52/10)	58.5 (22–87)	Tis–T4	G1–G3	Score = 3	RFS/CP	Binary variable	PCNA	Zymed (1 : 50)	Immunohistochemistry
Wang [29]	2001	China	49 (39/10)	61 (22–89)	NR	G1–G3	10%	RFS/CP	Binary variable	NR	NR	Immunohistochemistry
Miao [30]	1999	China	50	NR	Tis–T4	G1–G3	OC	RFS/OS/CP	Binary variable	Cyclin D1	Santa Cruz (1 : 100)	Immunohistochemistry
Shi et al. [31]	2003	China	82 (65/17)	58.7 (24–72)	Tis–T4	G1–G3	OC	RFS/CP	Binary variable	Cyclin D1	NR	Immunohistochemistry
Yang [32]	2005	China	69 (62/7)	61 (42–75)	Tis–T4	G1–G3	5%	RFS/CP	Binary variable	P27/nm23	NR	Immunohistochemistry
Wang et al. [33]	2013	China	45 (30/15)	65 (38–80)	NR	H/L	5%	RFS/CP	Binary variable	PTEN/P53	NR	Immunohistochemistry
Leng et al. [34]	2000	China	51 (43/8)	53.4 (28–72)	Tis–T3	G1–G3	OC	RFS/OS/CP	Binary variable	bcl-2	Santa Cruz (1 : 50)	Immunohistochemistry
Bai and Xiong [35]	2014	China	65 (50/15)	(57.7 ± 8.2)	Tis–T4	H/L	5%	CP	Binary variable	mfn2	Zymed (NR)	Immunohistochemistry
Wang et al. [36]	2000	China	75 (62/13)	58.5 (24–81)	Tis–T4	G1–G3	OC	CP	Binary variable	c-erbB-2, p53	Maxim (1 : 50)	Immunohistochemistry
Wang et al. [37]	2006	China	55 (35/20)	63 (24–75)	Tis–T4	G1–G3	10%	CP	Binary variable	hTERT, cyclin D1, RB	NR	Immunohistochemistry
Lu et al. [38]	2008	China	40 (30/10)	54.2 (37–79)	Tis–T4	G1–G3	10%	CP	Binary variable	p53, PCNA	NR (1 : 50)	Immunohistochemistry
Xie et al. [39]	2003	China	72 (56/16)	NR (29–78)	Tis–T4	G1–G3	5%	RFS/CP	Binary variable	Rb, cyclin D1	Zymed (1 : 50)	Immunohistochemistry
Qiu et al. [40]	2006	China	53 (46/7)	61 (25–83)	Tis–T4	G1–G3	15%	CP	Binary variable	NR	NR	Immunohistochemistry
Rebouissou et al. [41]	2012	France	89	NR	Ta–T1	G1–G3	Score = 3	RFS/PFS	Survival curve	FGFR3	NR	FISH
Abat et al. [42]	2014	Turkey	34 (30/4)	NR	T1–T4	Low and high	OC	PFS	Reported	p53	NR	FISH

M: male; F: female; RFS: recurrence-free survival; OS: overall survival; PFS: progression-free survival; DSS: disease-specific survival; CSS: cancer-specific survival; CP: clinicopathological parameters; OC: other criteria; NR: not reported; No.[b]: number of patients.

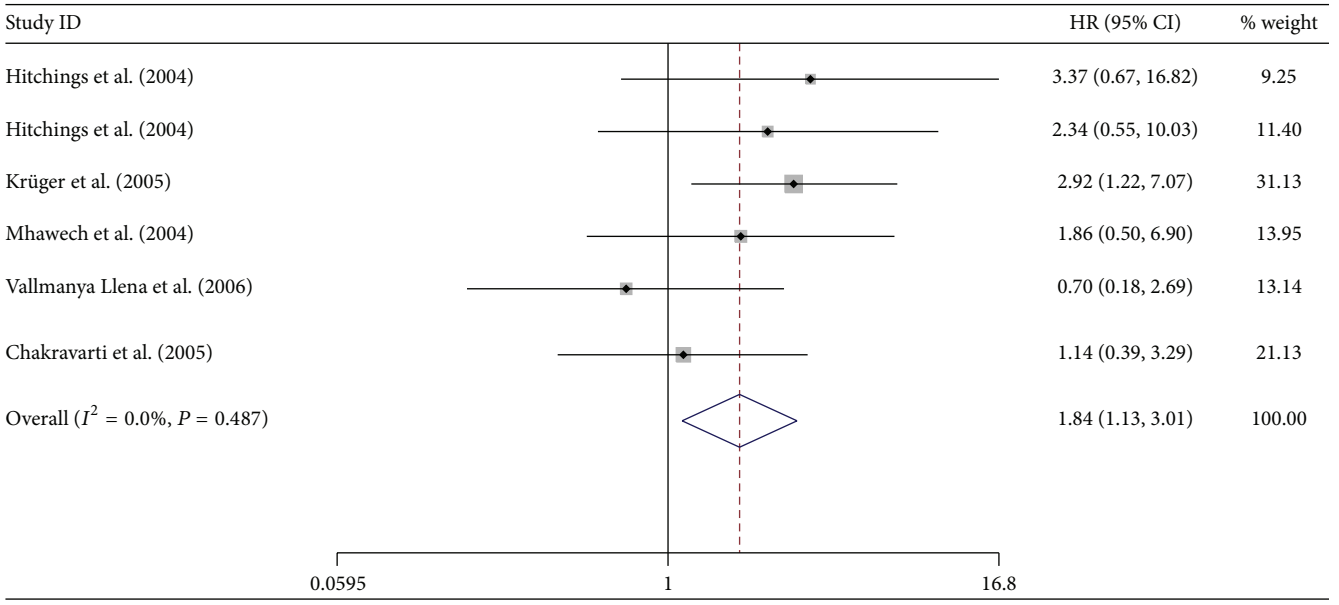


(a)



(b)

Figure 3: Continued.



(c)

Figure 3: Forrest plot of hazard ratio (HR) for the association of p16 with recurrence-free survival (RFS) (a), overall survival (OS) (b), and progression-free survival (PFS) (c) in patients with bladder cancer.

Subgroup analysis based on geographic region showed that low expression of p16 was associated with RFS in patients with bladder cancer both in Asia (HR = 1.44, 95% CI = 1.15~1.81, and $P = 0.002$) and in Europe (HR = 1.90, 95% CI = 1.13~3.19, and $P < 0.001$). The results of American studies (HR = 1.58, 95% CI = 0.77~3.25, and $P = 0.214$) need to be confirmed with larger sample sizes. The heterogeneity of Asian studies ($I^2 = 30.7\%$, $P = 0.173$) was lower than the overall heterogeneity, while that of Europe ($I^2 = 53.1\%$, $P = 0.037$) was higher, calculated with the random-effect model.

Subgroup analysis based on clinical stage suggested that the effect of p16 on RFS was associated with clinical stage (Tis-T1 group: HR = 1.96, 95% CI = 1.23~3.14, and $P < 0.001$; $I^2 = 55.5\%$, $P = 0.028$; Tis–T4 group: HR = 1.41, 95% CI = 1.12~1.77, and $P = 0.003$; $I^2 = 10.2\%$, $P = 0.348$).

Subgroup analysis based on histopathological grade showed that heterogeneity decreased from G1-G2 (HR = 4.12, 95% CI = 2.48~6.83, and $P < 0.001$; $I^2 = 0\%$, $P = 0.924$), G1–G3 (HR = 1.44, 95% CI = 1.18~1.75, and $P < 0.001$; $I^2 = 11.9\%$, $P = 0.323$), and G2-G3 (HR = 1.37, 95% CI = 0.78~2.42, and $P = 0.273$; $I^2 = 0\%$, $P = 0.802$), indicating that the effects of p16 on RFS in patients with bladder cancer were closely associated with pathological grade.

Subgroup analysis also showed an effect of cut-off value on the influence of p16 on RFS (cut-off value ≤ 10%: HR = 1.83, 95% CI = 1.34~2.51, and $P < 0.001$; $I^2 = 54.8\%$, $P = 0.009$; cut-off > 10%: HR = 1.34, 95% CI = 0.86~2.09, and $P = 0.003$; $I^2 = 10.2\%$, $P = 0.348$).

In addition, subgroup analysis of early-stage data from 430 subjects from eight studies also demonstrated that low expression of p16 significantly affected RFS in patients with early-stage (Ta–T1) bladder cancer (HR = 1.96, 95% CI = 1.23~3.14, and $P = 0.005$; $I^2 = 47.9\%$, $P = 0.088$).

3.3. Relationship between the Low Expression of p16 and OS in Patients with Bladder Cancer. A total of 425 subjects in eight studies were included in the final analysis of OS [9, 11, 14, 16, 19, 20, 30, 34], which showed that low expression of p16 was associated with decreased OS in patients with bladder cancer (HR = 1.70, 95% CI = 1.16~2.50, and $P = 0.007$), with no significant observed heterogeneity ($I^2 = 0\%$, $P = 0.584$) (Figure 3(b)).

Cumulative meta-analysis and sensitivity analysis indicated relatively low overall heterogeneity and no study with high sensitivity.

Subgroup analysis based on geographic area showed a subtle distinction between p16 expression and OS in patients with bladder cancer in Asia (HR = 1.61, 95% CI = 0.97~2.66, and $P = 0.065$; $I^2 = 0\%$, $P = 0.703$) and Europe (HR = 2.54, 95% CI = 1.05~6.15, and $P = 0.039$; $I^2 = 27.0\%$, $P = 0.250$).

Subgroup analysis was also performed based on clinicopathological stages. However, limitations of sample size led to the impossibility of determining if the effects of p16 expression on OS were associated with these parameters in patients with bladder cancer (Ta–T1 group: HR = 1.57, 95% CI = 0.32~7.75, and $P = 0.579$; $I^2 = 0\%$, $P = 0.750$; Ta–T4 group: HR = 1.59, 95% CI = 0.98~2.60, and $P = 0.061$; $I^2 = 3.1\%$, $P = 0.389$; T2–T4 group: HR = 1.96, 95% CI = 0.99~3.88, and $P = 0.053$; $I^2 = 52.1\%$, $P = 0.148$; low-grade group: HR = 1.41, 95% CI = 0.18~10.90, and $P = 0.742$; $I^2 = 0.0\%$, $P = 1.000$; G1–G3 group: HR = 1.82, 95% CI = 1.16~2.84, and $P = 0.009$; $I^2 = 3.9\%$, $P = 0.397$; high-grade group: HR = 1.41, 95% CI = 0.62–3.19, and $P = 0.409$; $I^2 = 0\%$, $P = 1.000$).

Subgroup analysis based on cut-off value indicated that the effects of p16 on OS in patients with bladder cancer were associated with cut-off value (cut-off value ≤ 10%: HR = 1.83,

95% CI = 1.17~2.86, and $P = 0.008$; $I^2 = 3.2\%$, $P = 0.006$; cut-off value > 10%: HR = 1.40, 95% CI = 0.66~2.96, and $P = 0.384$; $I^2 = 0\%$, $P = 0.951$).

3.4. Relationship between Low Expression of p16 and PFS in Patients with Bladder Cancer. A total of 470 subjects in seven studies were included in the ultimate analysis of PFS [5, 9, 10, 12, 19, 41, 42]. The results showed a correlation between low expression of p16 and poor PFS in patients with bladder cancer (HR = 2.18, 95% CI = 1.37~3.48, and $P = 0.001$), with low heterogeneity detected ($I^2 = 26.3\%$, $P = 0.219$).

Cumulative meta-analysis revealed no obvious characteristics because of the limited range of publication dates and the sample sizes.

Sensitivity analysis identified two studies [41, 42] as having the highest heterogeneities. Further investigation revealed that this heterogeneity was caused by different methods of measuring p16 (fluorescence in situ hybridization) and studying the influence of hemizygous or homozygous deletion of p16 on patient prognosis. These two studies were finally excluded because of their incompatible study objectives, leaving a total of 347 subjects from six studies in the final analysis of PFS. The results showed that low expression of p16 was correlated with poor PFS in patients with bladder cancer, and the heterogeneity was eliminated (HR = 1.84, 95% CI = 1.13~3.01, and $P = 0.015$; $I^2 = 0\%$, $P = 0.487$) (Figure 3(c)).

Despite a reduced sample size, subgroup analysis of the 347 subjects from five studies [5, 9, 10, 12, 19] demonstrated that the effects of p16 expression on PFS were affected by clinical stage (Ta–T1 group: HR = 2.09, 95% CI = 1.21~3.63, and $P = 0.002$; $I^2 = 0\%$, $P = 0.484$; T2–T4 group: HR = 1.14, 95% CI = 0.39~3.31, and $P = 0.810$; $I^2 = 0\%$, $P = 0.484$) and geographical location (Europe: HR = 2.09, 95% CI = 1.21~3.63, and $P = 0.002$; $I^2 = 0\%$, $P = 0.484$; America: HR = 1.14, 95% CI = 0.39~3.31, and $P = 0.810$).

Subgroup analysis based on cut-off value demonstrated some relationship between cut-off value and the influence of p16 expression on PFS (cut-off value ≤ 10%: HR = 2.61, 95% CI = 1.42~4.77, and $P = 0.002$; $I^2 = 0\%$, $P = 0.932$; cut-off value > 10%: HR = 0.95, 95% CI = 0.41~2.18, and $P = 0.896$; $I^2 = 0\%$, $P = 0.579$).

The results from 297 subjects with early-stage (Ta–T1) bladder cancer from four studies [5, 10, 12, 19] suggested that low expression of p16 was also significantly associated with poor PFS in early-stage bladder cancer (HR = 2.09, 95% CI = 1.21~3.63, and $P = 0.002$; $I^2 = 0\%$, $P = 0.484$).

3.5. Relationship between Low Expression of p16 and DSS/CSS in Patients with Bladder Cancer. A total of 187 subjects from three studies were included in the DSS/CSS analysis [9, 17, 18]; limitation of the sample size caused the impossibility of demonstrating an association between low expression of p16 and DSS/CSS (HR = 1.52, 95% CI = 0.85~2.71, and $P = 0.149$; $I^2 = 0\%$, $P = 0.825$).

3.6. Relationship between Low Expression of p16 and Clinicopathological Parameters in Patients with Bladder Cancer. The relationship between low expression of p16 and clinicopathological parameters [4, 10–13, 15–19, 21–40] was further explored by analysis of 30 studies including 1785 subjects. The results of statistical analyses were as follows: T2–T4/Ta–T1: OR = 3.13, 95% CI = 2.42~4.06, and $P < 0.001$; $I^2 = 1.4\%$, $P = 0.440$; T1/Ta: OR = 1.55, 95% CI = 0.87~2.76, and $P = 0.134$; $I^2 = 40.5\%$, $P = 0.152$; G3/G1-2 [43]: OR = 3.33, 95% CI = 2.51~4.42, and $P < 0.001$; $I^2 = 0\%$, $P = 0.519$; and H/L [44]: OR = 1.20, 95% CI = 0.69~2.33, and $P = 0.580$; $I^2 = 61.8\%$, $P = 0.011$; because of the high heterogeneity, a random-effects model was therefore applied. Meanwhile, these results demonstrated significant differences in the effects of low p16 expression in patients with bladder cancer between the two WHO clinical pathological grading methods in 1973 and 2004.

Analysis of the results for lymph node metastasis showed OR = 2.20, 95% CI = 1.26~3.83, and $P = 0.006$; $I^2 = 27.2\%$, $P = 0.240$. The small sample size caused the impossibility of demonstrating any significant influence of pathological parameters such as muscle invasion, tumor number (multiple/single), and tumor size on the effect of p16 expression (Table 2).

3.7. Retrospective Review. Three studies [6, 24, 25] were retrospectively reviewed because of differences between their prognosis results and the data required by the meta-analysis. As shown in Table 3, low expression of p16 was associated with poor prognosis in patients with bladder cancer. However, some of the P values were <0.05 because of the small sample sizes.

3.8. Publication Bias. Publication bias was detected by Begg's funnel plot and Egger's test (Figure 4). The points representing studies were symmetrically arranged in a funnel shape in the funnel plot, and the P values calculated from Egger's test with higher detection effectiveness were >0.05, indicating no publication bias. The only exception was for RFS; the funnel plot was asymmetrical and with a few points outside the funnel. Publication bias was also detected by Egger's test (G1–G3 group: $P = 0.031$; Asia group: $P = 0.020$), indicating potential publication bias in terms of RFS.

4. Discussion

p16, also known as tumor suppressor gene I (multiple tumor suppressor, MTS I), is located in 9p21 and is composed of two introns and three exons [45]. It is a key gene in cell cycle regulation, with its expression product being involved in the negative regulation of cell proliferation. Studies have shown that downregulation of p16 gene expression resulted in the loss of its inhibitory effects on CDK4/CDK6, which in turn may lead to malignant, abnormal cell proliferation and accelerated tumor development [7, 46, 47]. Elucidation of the relationship between low expression of p16 and prognosis and clinicopathology in patients with bladder cancer is therefore important for its early diagnosis, treatment, and prognosis.

Pan et al. performed a meta-analysis of the prognostic significance of abnormal p16 and p21 expression in bladder cancer in 2006 [48]. However, the current study analyzed a larger sample size; Pan et al.'s study included 12 articles with 975 cases, compared with 37 articles and 2246 cases

Table 2: Main meta-analysis results of p16 expression in patients with bladder cancer.

Analysis	No.[a] (No.[b])	HR (95% CI)	Z	P	Model	Heterogeneity		Publication bias	
						I^2%	P_{het}	Begg's P	Egger's P
RFS	18 (1032)	1.63 (1.36–1.94)	5.40	$P < 0.001$	F	42.6	0.029	0.405	0.246
Europe	8 (365)	1.90 (1.13–3.19)	2.43	$P = 0.003$	R	53.1	0.037	1.000	0.749
Asia	9 (547)	1.44 (1.15–1.81)	3.15	$P = 0.002$	F	30.7	0.173	0.348	0.020
America	1 (120)	1.58 (0.77–3.25)	1.24	$P = 0.214$	F	0.0	/	/	/
Ta–T1	8 (430)	1.96 (1.23–3.14)	2.82	$P = 0.005$	R	55.5	0.028	0.711	0.916
Ta–T4	10 (602)	1.41 (1.12–1.77)	2.96	$P = 0.003$	F	10.2	0.348	1.000	0.062
G1-G2	2 (75)	4.12 (2.48–6.83)	5.49	$P < 0.001$	F	0.0	0.924	1.000	/
G1–G3	14 (762)	1.44 (1.18–1.75)	3.50	$P < 0.001$	F	11.9	0.323	0.584	0.031
G2-G3	2 (95)	1.37 (0.78–2.42)	1.10	$P = 0.273$	F	0.0	0.802	1.000	/
Cut-off value (≤10%)	13 (741)	1.83 (1.34–2.51)	3.79	$P < 0.001$	R	54.8	0.009	0.583	0.297
Cut-off value (>10%)	5 (291)	1.34 (0.86–2.09)	1.28	$P = 0.200$	F	0.0	0.701	0.462	0.166
OS	9 (425)	1.70 (1.16–2.50)	2.71	$P = 0.007$	F	0.0	0.584	0.602	0.165
Europe	4 (167)	2.54 (1.05–6.15)	2.07	$P = 0.039$	F	27.0	0.250	1.000	0.289
Asia	4 (208)	1.61 (0.97–2.66)	1.85	$P = 0.065$	F	0.0	0.703	0.734	0.166
America	1 (50)	1.41 (0.62–3.19)	0.83	$P = 0.409$	F	0.0	/	/	/
Ta–T4	5 (230)	1.59 (0.98–2.60)	1.87	$P = 0.061$	F	3.1	0.389	1.000	0.232
Ta–T1	2 (122)	1.57 (0.32–7.75)	0.55	$P = 0.579$	F	0.0	0.750	1.000	/
T2–T4	2 (73)	1.96 (0.99–3.88)	1.94	$P = 0.053$	R	52.1	0.148	1.000	/
G1–G3	7 (353)	1.82 (1.16–2.84)	2.62	$P = 0.009$	F	3.9	0.397	/	/
L	1 (22)	1.41 (0.18–10.90)	0.33	$P = 0.742$	F	/	/	/	/
H	1 (50)	1.41 (0.62–3.19)	0.83	$P = 0.409$	F	/	/	/	/
Cut-off value (≤10%)	7 (278)	1.83 (1.17–2.86)	2.63	$P = 0.008$	F	3.2	0.402	0.764	0.185
Cut-off value (>10%)	2 (147)	1.40 (0.66–2.96)	0.87	$P = 0.384$	F	0.0	0.951	1.000	/
PFS	8 (470)	2.18 (1.37–3.48)	3.28	$P < 0.001$	F	26.3	0.219	0.174	0.325
IHC	6 (347)	1.84 (1.13–3.01)	2.44	$P = 0.015$	F	0.0	0.487	1.000	0.754
FISH	2 (123)	11.28 (2.45–51.83)	3.11	$P = 0.002$	F	0.0	0.718	1.000	/
Europe	5 (297)	2.09 (1.21–3.63)	2.62	$P = 0.009$	F	0.0	0.484	1.000	0.607
America	1 (50)	1.14 (0.39–3.31)	0.24	$P = 0.810$	F	/	/	/	/
Ta–T1	5 (297)	2.09 (1.21–3.63)	2.62	$P = 0.009$	F	0.0	0.484	1.000	0.607
T2–T4	1 (50)	1.14 (0.39–3.31)	0.24	$P = 0.810$	F	/	/	/	/
G1–G3	5 (297)	2.09 (1.21–3.63)	2.62	$P = 0.009$	F	0.0	0.484	1.000	0.607
H	1 (50)	1.14 (0.39–3.31)	0.24	$P = 0.810$	F	/	/	/	/
Cut-off value (≤10%)	4 (200)	2.61 (1.42–4.77)	3.10	$P = 0.002$	F	0.0	0.932	1.000	0.746
Cut-off value (>10%)	2 (147)	0.95 (0.41–2.18)	0.13	$P = 0.896$	F	0.0	0.579	1.000	/
DSS/CSS	3 (187)	1.52 (0.85–2.71)	1.42	$P = 0.155$	F	0.0	0.825	0.296	0.517
Clinicopathological parameters		OR (95% CI)							
Stage (T2–T4 versus Ta–T1)	19 (1231)	3.13 (2.42–4.06)	8.63	$P < 0.001$	F	1.4	0.440	0.529	0.377
Asia	14 (878)	3.41 (2.51–4.64)	7.87	$P < 0.001$	F	0.0	0.800	0.661	0.650
Europe	3 (277)	3.17 (1.79–5.60)	3.96	$P < 0.001$	F	63.7	0.064	1.000	0.994
America	2 (76)	1.15 (0.41–3.20)	0.26	$P = 0.796$	F	0.0	0.604	1.000	/
Stage (T1 versus Ta)	5 (374)	1.55 (0.87–2.76)	1.50	$P = 0.134$	F	40.5	0.152	0.806	0.402
Grade (G3 versus G1-2)	20 (1291)	3.33 (2.51–4.42)	8.32	$P < 0.001$	F	0.0	0.519	0.206	0.805
Asia	15 (895)	3.36 (2.44–4.63)	7.41	$P < 0.001$	F	18.6	0.246	/	/
Europe	3 (196)	2.62 (1.23–5.57)	2.50	$P = 0.013$	F	0.0	0.984	/	/
America	2 (200)	4.51 (1.61–12.61)	2.87	$P = 0.004$	F	0.0	0.659	/	/
Grade (H versus L)	8 (688)	1.20 (0.62–2.33)	0.55	$P = 0.580$	R	61.8	0.011	0.063	0.080
Lymph node metastasis (yes versus no)	5 (319)	2.20 (1.26–3.83)	2.77	$P = 0.006$	F	27.2	0.240	1.000	0.487
Muscle Invasive (yes versus no)	4 (248)	2.18 (0.72–6.62)	1.38	$P = 0.167$	R	71.8	0.014	0.497	0.998
Number of tumors (multiple versus single)	2 (166)	1.11 (0.43–2.85)	0.22	$P = 0.823$	F	0.0	0.984	1.000	/
Tumor size (>3 versus ≤3)	2 (193)	2.93 (0.40–21.36)	1.06	$P = 0.289$	R	79.2	0.028	1.000	/

RFS: recurrence-free survival; OS: overall survival; PFS: progression-free survival; DSS: disease-specific survival; CSS: cancer-specific survival; HR: hazard ratio; OR: odds ratio; CI: confidence interval; No.[a]: number of studies; No.[b]: number of patients; P_{het}: P for the heterogeneity; F: fixed-effect model; R: random-effect model; L: low grade; H: high grade.

Table 3: Relationship between low expression of p16 and other prognostic factors in patients with bladder cancer.

Author	Year	Nation	No.[b] (M/F)	Age	Stage	Grade	Cut-off value	Other related biomarkers	Measuring method	Antibody source (dilution)	Outcome	*P* value		
Jin et al. [24]	2006	USA	39 (25/14)	65 (42–84)	T2–T4	G1–G4	10%	P53, pRB	Immunohistochemistry	NR (1 : 50)	OS/PFS (2-year survival)	0.001	<0.001	
Tzai et al. [25]	2004	China (Taiwan)	65 (44/21)	61.5 (41–84)	T2–T4	G2-G3	Score = 4	P53, pRB	Immunohistochemistry	Santa Cruz (1 : 20)	PFS/DSS	0.74	0.49	
Yurakh et al. [6]	2006	Spain	55	NR	Ta–T4	G1–G3	10%	9p21 (P14, P15, P16)	Immunohistochemistry	Santa Cruz (1 : 500)	RFS/OS/PFS (3-year survival)	0.31	0.022	0.012

M: male; F: female; RFS: recurrence-free survival; OS: overall survival; PFS: progression-free survival; DSS: disease-specific survival; NR: not reported; No.[b]: number of patients.

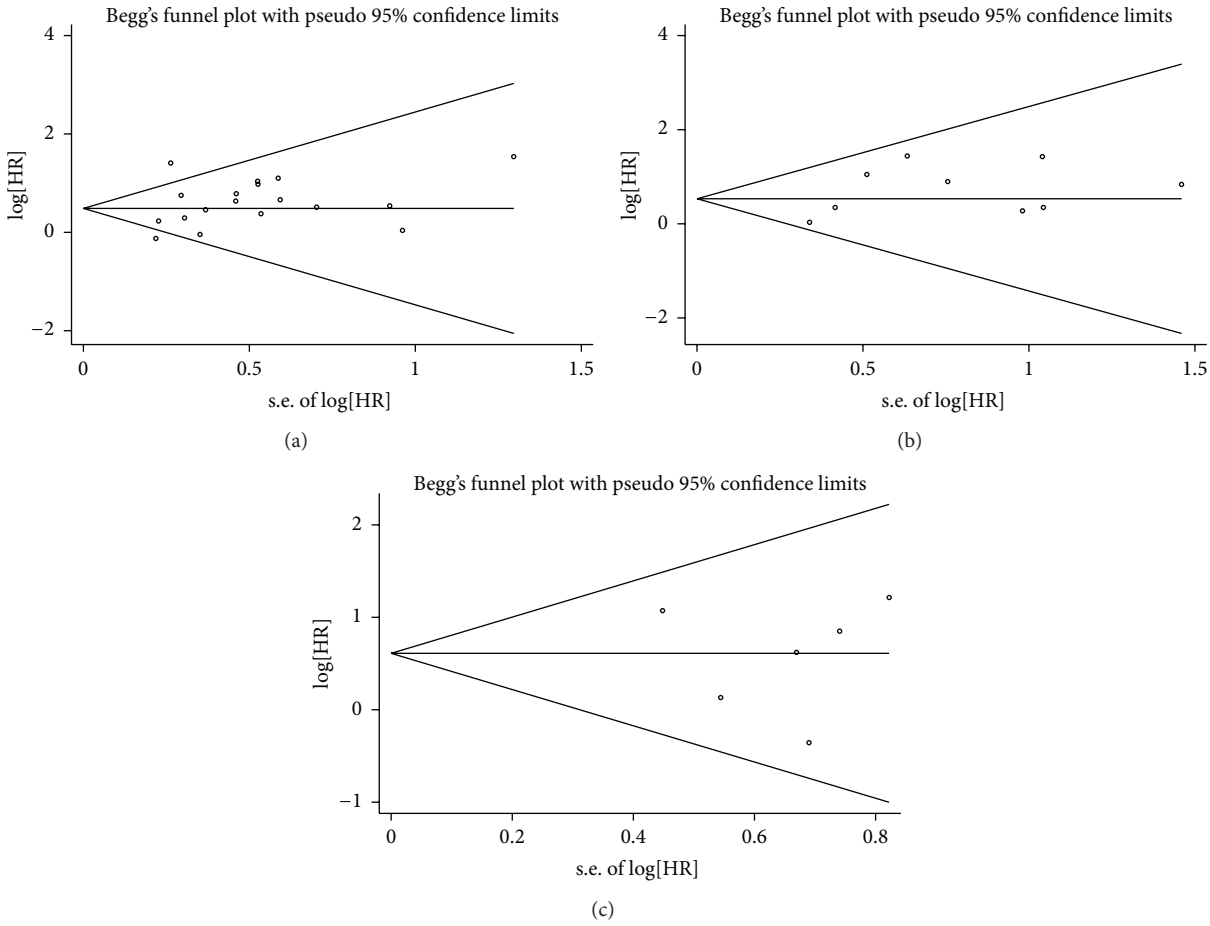


Figure 4: The funnel plot of the meta-analysis of the impact of p16 expression on recurrence-free survival (RFS) (a), overall survival (OS) (b), and progression-free survival (PFS) (c) in patients with bladder cancer.

in our study, leading to more accurate and reliable results. Secondly, Pan et al.'s study involved a number of mixed factors with no clear listing of each prognostic index or subgroup discussion. In contrast, the current study included subgroup analyses for the different indicators including RFS, PFS, OS, and DSS/CSS, allowing more thorough insights into the relationships between p16 expression and the prognostic and clinicopathological parameters in bladder cancer patients. Thirdly, Pan et al. found no association between p16 expression and prognosis in early Ta–T1 stage (stage I) bladder cancer, possibly because of the omission of the study by Krüger et al. [5], which explored the significance of p16 as an independent tumor predictive factor for the development of T1 bladder cancer, and demonstrated the important clinical value of low p16 expression in the early diagnosis and prognosis of patients with early-stage bladder cancer.

The current study systematically analyzed the relationships between p16 expression and prognostic index and clinicopathological parameters in patients with bladder cancer and showed that low expression of p16 was closely correlated with poor prognosis (Figure 5). However, the included studies varied in terms of study subjects, design, sample size, interventions, outcomes, time of study, and publication date. We used cumulative meta-analysis, sensitivity analysis, and subgroup analysis to explore the effects of the main variables in the included studies. Overall, the results confirmed that the relationship between low expression of p16 and prognosis in patients with bladder cancer was affected by clinicopathological stage, geographic origin of the study subjects, detection method, and cut-off values. Based on these findings, we further analyzed the relationships between p16 expression and clinicopathological parameters and demonstrated associations between low expression of p16 and clinical stage and lymph node metastasis, implying that the p16 gene tended to exert its regulatory effects during the early stage of bladder carcinogenesis. Low expression of p16 was also correlated with poor PFS and RFS in early-stage (Ta–T1) bladder cancer. These results thus confirmed an important role for p16 in the occurrence and development of bladder cancer. Meanwhile, through Phase I and II clinical trials, studies have revealed that CDK4/6 is an attractive target in p16 related pathway for anticancer therapy [49–51]. Furthermore, previous study also suggested that p16 functional peptide, as a molecular targeting agent, showed effective reactions for the treatment of renal cell carcinoma [52].

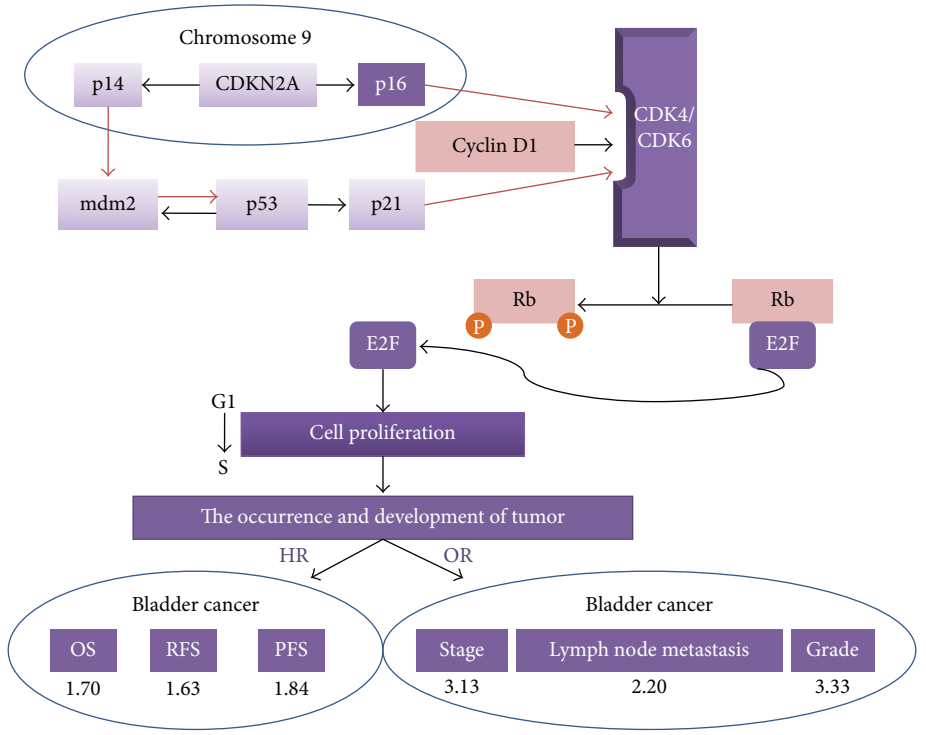


Figure 5: Our results illustrated and improved the relationship between p16 and prognosis, as well as clinicopathological features.

The results of these researches and our current meta-analysis had the effect of mutual authentication. Therefore, a better understanding of the mechanism underlying the development and progression of bladder cancer may play a significant role in prevention, target therapy, and prognosis, particularly if more sensitive and specific correlative biomarkers can be discovered and verified.

The current study had some limitations. First, tumors are the result of both environmental and genetic factors, and p16 may thus be only one of several factors involved in the whole process of bladder carcinogenesis. Secondly, heterogeneity may result from differences in intervention measures (surgery, radiotherapy, chemotherapy, or combination), immunohistochemical techniques (different antibodies, evaluation standards, etc.), and the HR extraction methods used in the included studies. Finally, the exclusion of articles because of language barriers and of studies that were not published because of a lack of sufficient data may have led to potential publication bias.

In conclusion, the results of the current study provide evidence for a relationship between p16 expression and prognosis and clinicopathological features in patients with bladder cancer. The results of this meta-analysis will help to inform about the development of clinical guidelines promoting best medical care for patients with bladder cancer. Further studies are required to investigate the combined influence of genetic and environmental factors on the development and progression of bladder cancer.

Abbreviations

RFS: Recurrence-free survival
OS: Overall survival
PFS: Progression-free survival
DSS: Disease-specific survival
CSS: Cancer-specific survival
HR: Hazard ratio
OR: Odds ratio
CI: Confidence interval.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Xiaoning Gan, Xiaomiao Lin, Rongquan He, Xinggu Lin, Hanlin Wang, Liyan Yan, Hong Zhou, and Hui Qin performed the literature search, data extraction, and statistical analysis and drafted the paper. Gang Chen supervised the literature search, data extraction, and analysis and reviewed the paper. Xiaoning Gan, Xiaomiao Lin, Rongquan He, Xinggu Lin, Hanlin Wang, Liyan Yan, Hong Zhou, Hui Qin, and Gang Chen read and approved the final paper.

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Research Article

HER2 Protein Overexpression and Gene Amplification in Plasmacytoid Urothelial Carcinoma of the Urinary Bladder

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Aim. HER2 overexpression has been reported in a minority of urothelial carcinomas, but little is known about HER2 protein expression and gene alterations in plasmacytoid urothelial carcinoma, a rare and aggressive variant. The aim of this study was to clarify the HER2 status in plasmacytoid urothelial carcinomas. *Methods*. Six cases of plasmacytoid urothelial carcinoma were included, in which we evaluated HER2 protein expression by immunohistochemistry (IHC) and *HER2* gene amplification by fluorescence *in situ* hybridization (FISH). *Results*. The patients' ages ranged from 57 to 83 years (mean age, 71 years). Five patients were male and one was female. The ratio of the plasmacytoid component ranged from 30% to 100% (mean, 77%). HER2 expression score was 3+ in 4 cases, 2+ in one case, and negative in one case. HER2 gene amplification was positive in 3 cases, of which 2 cases showed a 3+ HER2 IHC score but one case was negative for HER2 IHC. Another 2 cases showed equivocal HER2 FISH results, and one remaining case was negative for HER2 FISH. *Conclusion*. Our observation that plasmacytoid urothelial carcinomas frequently demonstrated HER2 protein overexpression provides supporting evidence that HER2 may be a potential therapeutic target for plasmacytoid urothelial carcinoma.

1. Introduction

Many histologic variants of urothelial carcinoma have been described [1–3]. Variants of urothelial carcinoma show different prognosis [4]. Among these variants, plasmacytoid urothelial carcinoma is a rare and aggressive variant of urothelial carcinoma and is associated with poor prognosis [5–10].

Human epidermal growth factor receptor type 2 (HER2) is a transmembrane receptor tyrosine kinase, and its coding gene is located on chromosome band 17q21 [11]. HER2 overexpression in cancer is associated with poor prognosis in various cancers [12], and anti-HER2 therapy is well established for HER2 overexpressing breast cancers and gastric cancers [13, 14]. Recently, HER2 protein overexpression and gene amplification have been reported in urothelial carcinomas, and some studies have shown the prognostic significance of HER2 overexpression or gene amplification in urothelial carcinoma [15–18]. As a result, HER2 is being considered as a new therapeutic target for urothelial carcinomas [19, 20].

The frequency of HER2 overexpression or gene amplification in urothelial carcinoma is approximately 10% [15, 19, 21]. Among the known variants of urothelial carcinoma, the micropapillary variant of urothelial carcinoma frequently showed HER2 protein overexpression and HER2 gene amplification [22]. Additionally, HER2 gene amplification in the micropapillary variant of urothelial carcinoma is associated with poor outcome [23]. A recent study also revealed the different incidence of HER2 gene amplification among the variants of urothelial carcinoma [24]. However, little is known about HER2 expression and gene amplification in plasmacytoid urothelial carcinoma. Only one study showed HER2 protein overexpression in 2 cases of plasmacytoid urothelial carcinoma with concurrent micropapillary component [25].

In the present study, we investigated HER2 protein expression and HER2 gene amplification in plasmacytoid urothelial carcinomas.

Table 1: Clinicopathologic characteristics of plasmacytoid urothelial carcinomas.

Case	Age/sex	Presenting symptoms	Operation	TNM stage	Plasmacytoid component	Other components	Follow-up status
1	64/M	Hematuria	TUR	IV	30%	CONV	LOF, after 16 mo
2	83/M	Hematuria	RC	IV	80%	CONV, MP	DOD, 28 mo
3	69/M	Urgency	RC	III	80%	CONV	DOD, 10 mo
4	73/M	Hematuria	RC	IV	100%	None	LOF, after 3 mo
5	57/M	Hematuria	RC	IV	100%	None	AWD, 19 mo
6	82/F	Hematuria	TUR	I	70%	CONV	AWD, 1 mo

AWD indicates alive with disease; CONV, conventional type; DOD, died of disease; F, female; LOF, loss of follow-up; M, male; MP, micropapillary type; RC, radical cystectomy; TUR, transurethral resection.

2. Materials and Methods

2.1. Case Selection and Tissue Microarray (TMA) Construction. We searched the computerized database of the Department of Pathology, Seoul National University Hospital, and we found six cases that had been diagnosed as plasmacytoid urothelial carcinoma in bladder tumor specimens between 2005 and July 2015. We reviewed the hematoxylin and eosin-stained slides to confirm the adequacy of the diagnosis and various pathologic parameters, including the percentage of plasmacytoid urothelial carcinoma component, the presence of concomitant conventional urothelial carcinoma and other variants, and the TNM stage. We collected the clinical data and pathologic information from the medical records and pathology reports. A TMA block was prepared from formalin-fixed paraffin-embedded tissue blocks (SuperBioChips Laboratories, Seoul, Korea). Two to seven cores containing various tumor areas were obtained from each case. This study was approved by the Institutional Review Board (IRB) of Seoul National University Hospital.

2.2. Immunohistochemistry (IHC). Immunohistochemical staining for HER2 was performed using the HercepTest™ kit (Dako, Glostrup, Denmark) according to manufacturer's protocols. 4 μm thick sections taken both from original full tumor blocks and the TMA block were used for HER2 IHC. HER2 protein expression was scored as 0, 1+, 2+, and 3+ according to the ASCO/CAP 2013 HER2 test guideline [26]. HER2 IHC 3+ was considered to be HER2-positive, IHC 2+ HER2-equivocal, and IHC 0 and 1+ HER2-negative [26]. Additional immunohistochemical staining for cytokeratin (CK) 7 (clone OV-TL 12/30; Dako), CK20 (clone Ks20.8; Dako), p53 (clone DO7; Dako), p63 (clone 4A4; Ventana), and CD138 (clone 5F7; Novocastra) was also performed on sections taken from the TMA block. All IHC were performed using Ventana Benchmark XT automated staining system (Ventana Medical Systems, Tucson, AZ).

2.3. Fluorescence In Situ Hybridization (FISH). HER2 gene amplification was examined by dual-color FISH analysis using PathVysion HER2 DNA Probe Kit (Abbott, Abbott Park, IL) on TMA sections according to the manufacturer's protocols. FISH results were analyzed by counting the fluorescence signals in 20 malignant cells. For each case, the average *HER2* copy number and the ratio of *HER2* signals to chromosome 17 centromere (CEP17) signals were calculated. HER2 positivity by FISH was defined as an average *HER2* copy number ≥6.0 or *HER2*/CEP17 ratio ≥2.0 according to the ASCO/CAP 2013 HER2 test guideline [26]. The cases showing *HER2*/CEP17 ratio <2.0 with average *HER2* copy number ≥4.0 and <6.0 were regarded as HER2-equivocal, and the cases showing *HER2*/CEP17 ratio <2.0 with average *HER2* copy number <4.0 were regarded as HER2-negative.

The results of the HER2 test were considered positive when the tumor specimens showed HER2 IHC 3+ or positive *HER2* gene amplification by FISH.

3. Results

3.1. Clinical and Pathologic Characteristics of Plasmacytoid Urothelial Carcinomas. Six plasmacytoid urothelial carcinoma cases were included in this study, of which 5 were male and one was female. Table 1 summarizes the clinical and pathologic features of these 6 cases. The mean age was 71 years (range, 57–83 years). The initial presenting symptom was hematuria in 5 cases and urgency in one case. Four patients had TNM stage IV disease, one stage III disease, and one stage I disease. The proportion of the plasmacytoid component ranged from 30% to 100% (mean, 77%) (Figures 1(a), 1(d), and 1(g)). Two cases consisted entirely of plasmacytoid components (Cases 4 and 5), and remaining four cases contained conventional or micropapillary urothelial carcinoma components (Table 1).

3.2. HER2 Protein Expression and HER2 Gene Amplification. The HER2 IHC and FISH results are summarized in Table 2.

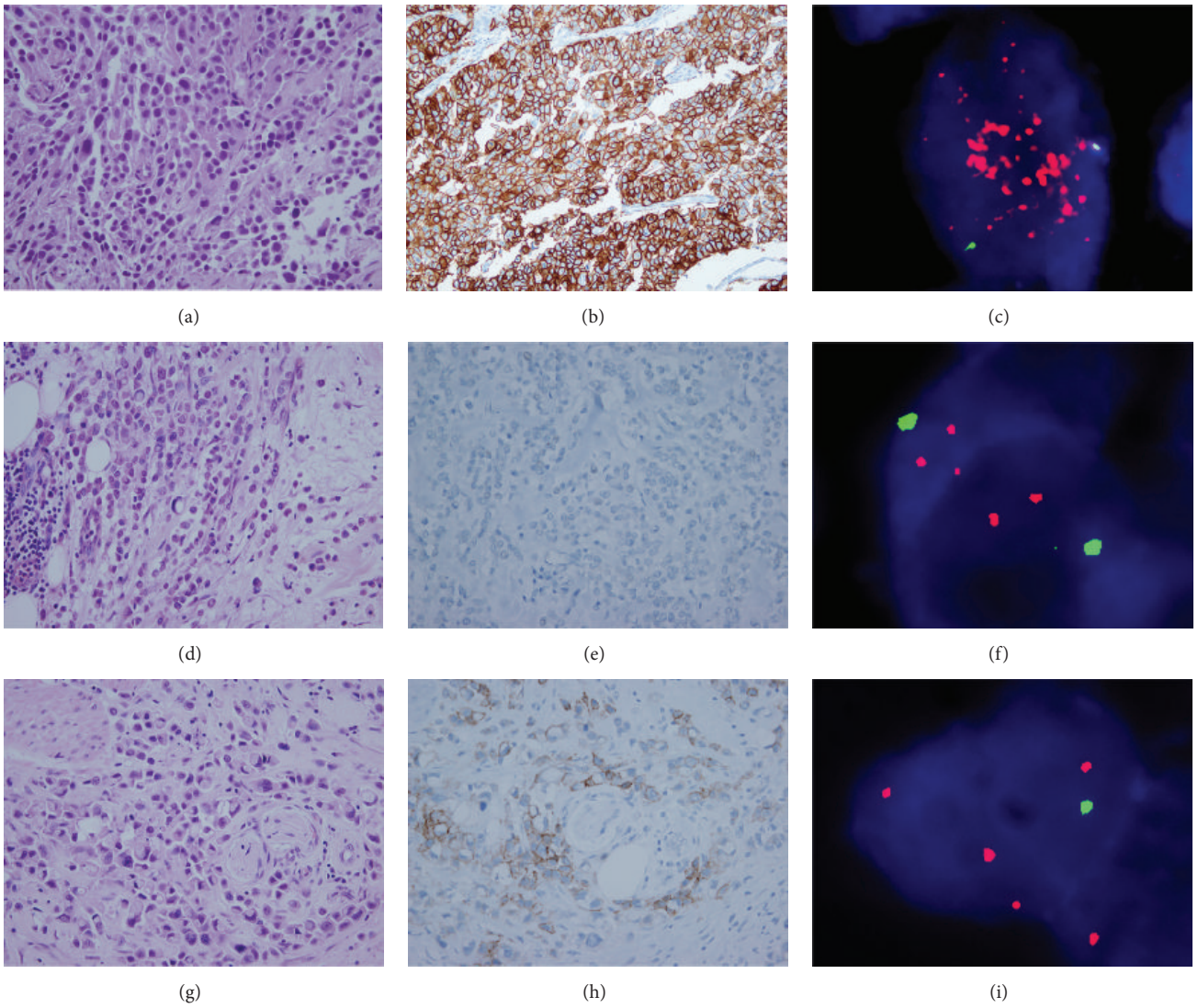


Figure 1: Plasmacytoid component of case 1 (a) showed diffuse 3+ HER2 IHC positivity (b) and *HER2* gene amplification (*HER2*/CEP17 ratio 5.8) by FISH (c). Case 3 (d) was negative for HER2 by IHC (e), but the *HER2* gene was amplified (*HER2*/CEP17 ratio 2.06) (f). Case 4 (g) showed 2+ HER2 by IHC (h) and equivocal *HER2* FISH results (*HER2*/CEP17 ratio 1.81 and average *HER2* gene copy number 4.9) (i).

Four cases showed IHC 3+ by HER2 IHC, and in these four cases, most tumor cells with plasmacytoid morphology showed intense membranous HER2 staining (Figure 1(b)). One case had equivocal HER2 immunostaining (IHC 2+) (Figure 1(h)) and one remaining case did not stain for HER2 (IHC 0) (Figure 1(e)). By *HER2* FISH, three cases showed a *HER2*/CEP17 ratio ≥2.0 (Figures 1(c) and 1(f)). Another two cases had *HER2*/CEP17 ratios <2.0 but with an average *HER2* copy number ≥4.0 and were considered to be equivocal for *HER2* gene amplification. One case (case 3) was negative for HER2 by IHC but FISH demonstrated positive *HER2* gene amplification (Figures 1(h) and 1(i)). Overall, the HER2 test was positive in five cases and equivocal in one case (Table 2). HER2 IHC results of conventional or micropapillary components were identical to those of plasmacytoid components. *HER2* gene amplification of conventional or micropapillary components was positive in 2 cases and negative in remaining two cases (Table 2).

3.3. Immunohistochemical Staining. Immunohistochemical staining results are detailed in Table 3. CK7 was positive in five cases, CK20 in six cases, and p63 in four cases. CD138, an immunohistochemical marker for plasma cells, was positive in all six cases (Figure 2). p53 was diffusely positive in one case and focally positive in four other cases.

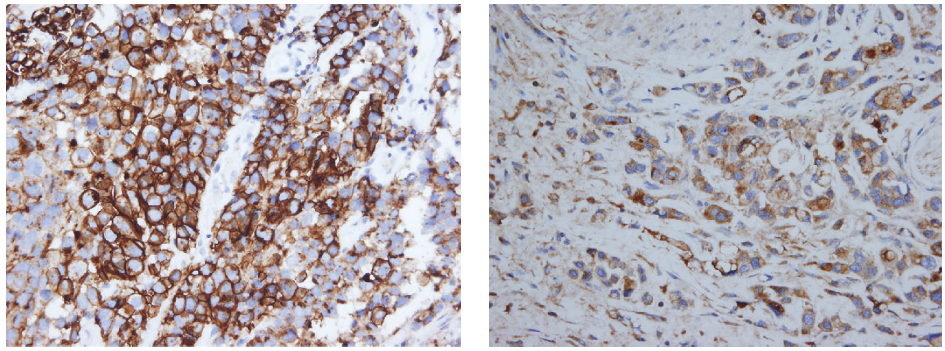
4. Discussion

Urothelial carcinoma is the most common cancer of the urinary bladder. Many histologic variants of urothelial carcinoma have been reported, and these variants showed clinicopathological features distinct from those of conventional

Table 2: HER2 IHC and FISH results.

Case	Component	HER2 IHC	*HER2* gene amplification	*HER2*/CEP17 ratio	Average *HER2* copy number
1	P	Positive (3+)	Positive	5.8	20.0
	CONV	Positive (3+)	Positive	3.03	9.0
2	P	Positive (3+)	Positive	3.61	9.8
	MP	Positive (3+)	Positive	2.2	9.0
	CONV	Positive (3+)	Positive	2.13	7.5
3	P	Negative (0)	Positive	2.06	4.9
	CONV	Negative (0)	Negative	1.27	2.0
4	P	Equivocal (2+)	Equivocal	1.81	4.9
5	P	Positive (3+)	Negative	1.68	3.5
6	P	Positive (3+)	Equivocal	1.61	4.5
	CONV	Positive (3+)	Negative	1.07	3.2

P, plasmacytoid component; CONV, conventional component; MP, micropapillary component.



(a) (b)

Figure 2: Case 1 (a) and case 4 (b) showed strong diffuse immunopositivity for CD138.

Table 3: Immunohistochemical staining results.

Case	p63	CK7	CK20	p53	CD138
1	P	P	P	Focal strong P	P
2	P	P	P	Negative	P
3	N	N	Focal P	Focal moderate P	P
4	N	P	Focal P	Focal strong P	P
5	P	P	P	Diffuse strong P	P
6	P	P	P	Focal moderate P	P

P indicates positive; N, negative.

urothelial carcinoma [1, 2, 4]. Plasmacytoid urothelial carcinoma is a rare variant of urothelial carcinoma characterized by tumor cells that resemble plasma cells [5, 6].

In the present study, we evaluated the HER2 protein expression and *HER2* gene amplification in six cases of plasmacytoid urothelial carcinoma. Our study revealed that plasmacytoid urothelial carcinoma frequently showed HER2 protein overexpression and *HER2* gene amplification. Five cases out of six were considered to be HER2-test-positive and one was considered as equivocal according to the ASCO/CAP 2013 HER2 test guideline. Little is known about the HER2 status in plasmacytoid urothelial carcinoma. Only one case report showed HER2 protein expression by IHC in two plasmacytoid urothelial carcinoma cases, of which one case was HER2 IHC 3+ and the other one 2+ [25]. There has been no study about *HER2* gene amplification in plasmacytoid urothelial carcinoma. To the best of our knowledge, our study is the first to demonstrate that the *HER2* gene is frequently amplified in plasmacytoid urothelial carcinoma.

HER2 gene amplification is found in a small subset of urothelial carcinomas and is related to poor prognosis [15, 16]. Among the variants of urothelial carcinoma, the micropapillary variant of urothelial carcinoma showed a higher incidence of *HER2* gene amplification and HER2 protein overexpression than conventional urothelial carcinoma. Ching et al. revealed that 68% of micropapillary urothelial carcinoma (13/19) overexpressed HER2 protein (2+ to 3+ by HER2 IHC), and 42% (8/19) showed gene amplification [22]. Although the number of cases was limited, our study showed that the majority of cases of plasmacytoid urothelial carcinoma were also positive in the HER2 test.

HER2 overexpression and gene amplification have been known to be related to the aggressive behavior of various cancers including urothelial carcinoma [12, 15, 16, 18]. Micropapillary urothelial carcinoma is also an aggressive variant, and HER2 gene amplification may be related to the poor prognosis of this variant [23]. In this regard, HER2 overexpression and gene amplification in plasmacytoid urothelial carcinoma may be associated with the aggressive behavior of this tumor.

In this study, two cases (cases 3 and 5) showed discrepancies between the results of HER2 IHC and FISH. Some previous studies also showed the discrepancies between HER2 IHC and FISH in some urothelial carcinoma cases [22, 23]. Furthermore a previous study described that there was no strong association between HER2 protein overexpression and gene amplification in contrast to breast cancer [27]. This study also revealed the discrepancies between the results of HER2 IHC and FISH in two out of six plasmacytoid urothelial carcinomas. Further study will be needed to elucidate these discrepancies in urothelial carcinoma.

HER2 is a well-established therapeutic target in some cancers characterized by HER2 protein overexpression or gene amplification [13, 14]. Targeted therapy against HER2 has been attempted in patients with urothelial carcinoma showing HER2 gene amplification [20, 28]. Plasmacytoid urothelial carcinoma is a very aggressive variant [4]. Although neoadjuvant chemotherapy for this tumor led to improvement of the pathological stage of patients in a study, the outcome was dismal [8]. The results of our study suggested that HER2 may be a good candidate for targeted therapy of plasmacytoid urothelial carcinoma.

CD138 is known to be a marker for plasma cells, and variable expression of CD138 in plasmacytoid urothelial carcinoma has been reported in previous studies [5, 6, 10, 25]. CD138 is also expressed in urothelial carcinoma *in situ*, conventional urothelial carcinoma, and even normal urothelium [29, 30]. Our study confirmed the expression of CD138 in plasmacytoid urothelial carcinoma, and the incidence was 100% (6/6 cases).

5. Conclusion

In summary, our study demonstrated the frequent occurrence of HER2 protein overexpression and gene amplification in plasmacytoid urothelial carcinomas. This result provides supporting evidence that HER2 may be a potential therapeutic target for the control of this aggressive tumor.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

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Research Article

Network Biomarkers of Bladder Cancer Based on a Genome-Wide Genetic and Epigenetic Network Derived from Next-Generation Sequencing Data

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Epigenetic and microRNA (miRNA) regulation are associated with carcinogenesis and the development of cancer. By using the available omics data, including those from next-generation sequencing (NGS), genome-wide methylation profiling, candidate integrated genetic and epigenetic network (IGEN) analysis, and drug response genome-wide microarray analysis, we constructed an IGEN system based on three coupling regression models that characterize protein-protein interaction networks (PPINs), gene regulatory networks (GRNs), miRNA regulatory networks (MRNs), and epigenetic regulatory networks (ERNs). By applying system identification method and principal genome-wide network projection (PGNP) to IGEN analysis, we identified the core network biomarkers to investigate bladder carcinogenic mechanisms and design multiple drug combinations for treating bladder cancer with minimal side-effects. The progression of DNA repair and cell proliferation in stage 1 bladder cancer ultimately results not only in the derepression of miR-200a and miR-200b but also in the regulation of the TNF pathway to metastasis-related genes or proteins, cell proliferation, and DNA repair in stage 4 bladder cancer. We designed a multiple drug combination comprising gefitinib, estradiol, yohimbine, and fulvestrant for treating stage 1 bladder cancer with minimal side-effects, and another multiple drug combination comprising gefitinib, estradiol, chlorpromazine, and LY294002 for treating stage 4 bladder cancer with minimal side-effects.

1. Introduction

Bladder cancer is still one of the most common cancers worldwide. Single gene markers have been proposed for improving cancer treatment [1]. However, single gene markers cannot overcome treatment side-effects because the markers are not implicated in genome-wide networks, and the analysis of a genome-wide network is a complicated issue from a systems biology perspective. The rapid development of molecular biology techniques has produced a great deal of high-throughput experimental data, including genome-wide microarray data, genome-wide methylation profiles, next-generation sequencing (NGS) data, microRNA (miRNA) profiles, genetic sequences, protein abundance data, and drug response genome-wide microarray data. These kinds of omics data provide an opportunity to design multiple drug combinations for the treatment of bladder cancer by applying the network biomarkers identified by systems biology.

To date, genetic regulation systems, including protein-protein interaction networks (PPINs) and gene regulatory networks (GRNs), have been applied to analyze the functional mechanisms behind human aging and cancer [2, 3]. We now know that epigenetic alterations are much more rapid and adaptive with regard to influencing genome-wide gene expression than genetic changes [4]. Rapid and slow response mechanisms, that is, epigenetic alterations and genetic changes, respectively, coordinate an efficient and robust system. Epigenetic regulation, including DNA methylation and histone modification, results in potentially reversible alterations in gene expression that do not involve permanent changes to the DNA sequence. miRNAs that are influenced by aberrant epigenetic regulation also mediate the regulation of

gene expression [5]. It has been found that DNA methylation directly affects the binding affinities of miRNAs, RNA polymerase, and transcription factors (TFs) [6] and indirectly influences protein-protein interactions (PPIs) [7]. Methylation analysis of human genomic DNA in 12 tissues revealed that DNA methylation profiles are tissue-specific [8]. Therefore, omics data and systems biology methods [9–11] are required to unravel the mechanisms underlying carcinogenesis from the complex molecular biology and design anticancer drugs for the treatment of bladder cancer.

The Human Genome Project (HGP) has identified 30,000–40,000 genes in human DNA, including miRNAs. The genes, proteins, and their associations, miRNA regulation, and DNA methylation constitute the integrated genetic and epigenetic genome-wide network (IGEN), which coordinates cellular responses. PPIN in human lung cancer [12] and GRN in human aging [13] of the genes with significant expression differences between cancer cells (or aged people) and normal cells (or young people) have been identified for the extraction of the core network biomarkers according to the estimated association abilities between TFs (or upstream proteins) and target genes (or target proteins). Aging is associated with cancer [14]. The association abilities estimated by the network models assume that the binding affinities of TFs (or upstream proteins) to target genes (or proteins) are the same. According to a recent study in primary human somatic and germline cells [6], the impact of the binding affinities of miRNAs, RNA polymerase, and TFs on gene expression is mediated by DNA methylation. According to the available genome-wide methylation profiles and NGS data for bladder cancer in The Cancer Genome Atlas (TCGA), DNA methylation and miRNA regulation can be also characterized by the GRN model to identify the genome-wide IGEN. In this study, we identified the IGENs in normal bladder cells and bladder cancer cells and then investigated the impact of epigenetic regulation and miRNA regulation on bladder carcinogenesis by comparing the IGEN in normal bladder cells with that in bladder cancer cells.

Although a genome-wide IGEN can be identified based on well-defined system identification techniques [2, 12], the mean by which the core network biomarkers are extracted from the identified genome-wide network is still an important issue. The total association capabilities of a single node can affect the contribution it makes to its neighbors. However, the genome-wide IGEN including transcriptional gene regulations, miRNA regulations, and PPIs constitutes a genome-wide network structure. The contribution made by one node to its neighbors is not sufficient to explain its impact on a genome-wide scale network of bladder cells. In this study, we applied a principal genome-wide network projection (PGNP) based on principal component analysis (PCA) to identify core network biomarkers in bladder carcinogenesis, with the objective of extracting the most significant part from a genome-wide network structure. Because the drug response genome-wide microarray data are now available [15], we analyzed the drug response microarray data of the core network biomarkers to design multiple drug combinations with minimal side-effects for bladder cancer treatment. Therefore, the identified core network biomarkers could provide an opportunity to design such drug combinations for bladder cancer treatment. Furthermore, it has been reported that aging (over 45 years old) and smoking are two major risk factors for bladder carcinogenesis [16]. Therefore, we used the core network biomarkers to elucidate the cellular mechanisms by which aging and smoking elevate bladder cancer risk through epigenetic regulation, miRNA regulation, and signaling pathways.

According to the strategy shown in the flowchart (Figure 1), we integrated omics data, including genome-wide methylation profiles, NGS expression data, miRNA profiles in TCGA, drug response genome-wide microarray data in the Connectivity Map (CMAP) [15], drug-gene interaction data in the Drug Gene Interaction Database (DGIdb) [17], miRNA-target gene association data in TargetScan [18], PPIs in BioGRID, transcription regulations in the Human Transcriptional Regulation Interactions database (HTRIdb) [19], the Integrated Transcription Factor Platform (ITFP) [20], and the TRANSFAC [21], biological processes and pathways in a gene ontology (GO) database, the National Center for Biotechnology Information (NCBI) Entrez Gene database, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [22]. We used miRNA-target gene association data, PPIs, and transcription regulations to build the candidate IGEN for general molecular mechanisms. We then constructed a regression IGEN model to characterize the molecular mechanisms including miRNA regulation, PPIs, transcription regulation, and DNA methylation in cells. To prune the false positive connections in the candidate IGEN and identify the model parameters of the IGEN in the real human bladder cells, we used methylation profiles, NGS expression data, and miRNA profiles in normal bladder cells and stage 1 and stage 4 bladder cancer cells. We then applied the constrained least squares method and the Akaike information criterion (AIC) [23], a system order detection method, to prune the false positive connections for obtaining the real IGENs in the three stages of human bladder carcinogenesis. The three genome-wide real IGENs in normal bladder cells and stage 1 and stage 4 bladder cancer cells were then projected into the three core networks of the three stages of bladder carcinogenesis, respectively. Because the core networks contain the identified signal transduction pathways, that is, the receptors and TFs of the core network can be directly or indirectly connected by the core proteins/TFs, the proteins/TFs, and the corresponding genes that participate in the identified signaling pathways of the core networks are considered as the core network biomarkers for normal and cancerous cells, respectively. The miRNAs with very different connections in regulating the genes of the core network biomarkers between two cells are also involved in the core network biomarkers. By comparing the identified connections of the IGENs, we investigated how the connection changes of the core network biomarkers from normal bladder cells to stage 1 bladder cancer cells and from stage 1 bladder cancer cells to stage 4 bladder cancer cells contribute to bladder carcinogenesis.

We also investigated how the module network of the core network biomarkers, including the KEGG pathways and biological processes, participates in bladder carcinogenesis.

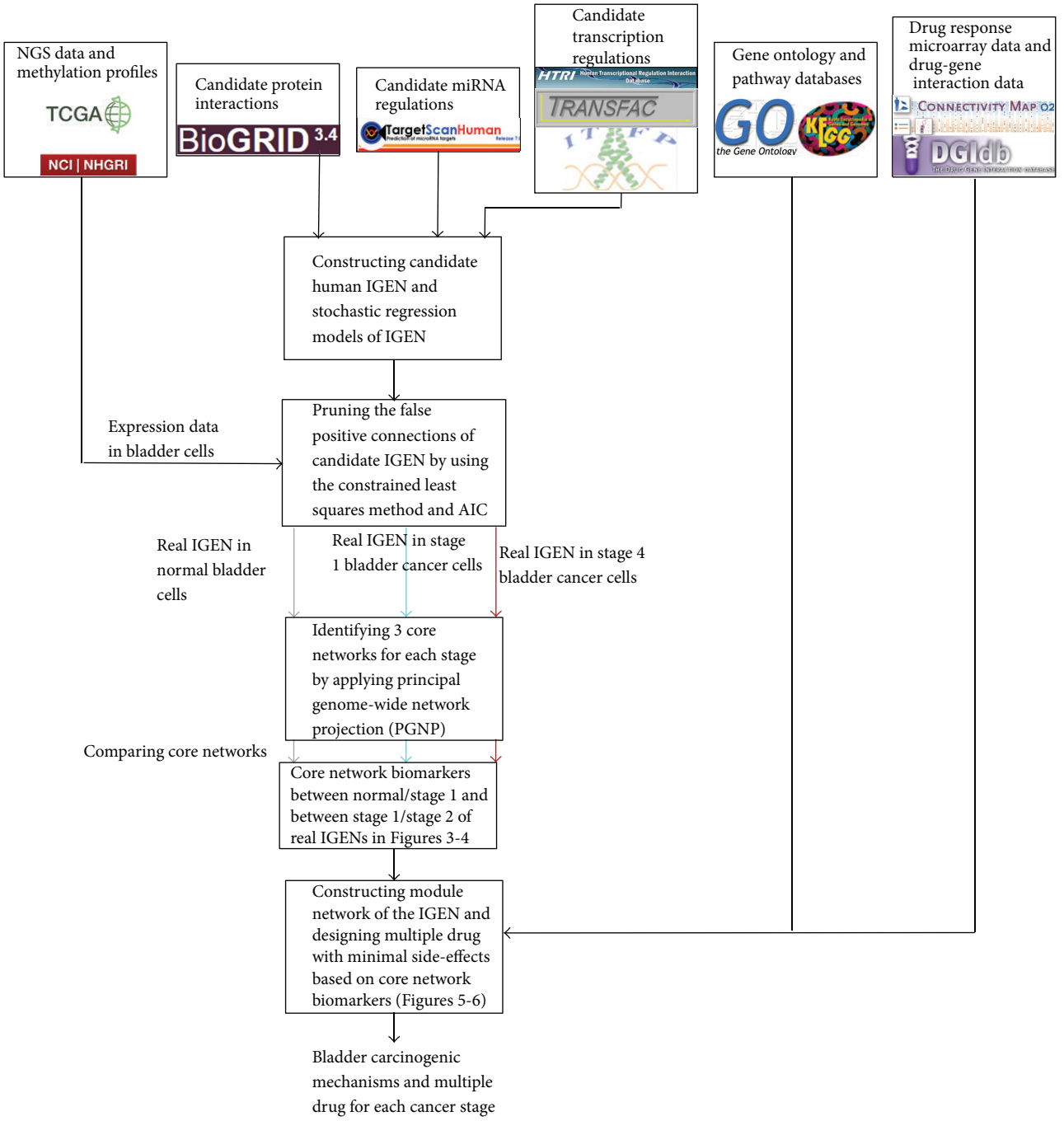


FIGURE 1: Flowchart of the proposed method for constructing the core network biomarkers and identifying bladder carcinogenesis mechanisms.

According to the information on the biological processes and signaling pathways in the GO database, the NCBI Entrez Gene database, and the KEGG pathway database, the roles of the TFs/proteins in the core network biomarkers are projected into three pathways: the SUMOylation, ubiquitination, and proteasome (SUP) pathway; the tumor necrosis factor (TNF) signaling pathway; and the endoplasmic reticulum (ER) signaling pathway. The roles of the downstream genes in the core network biomarkers are projected into three biological processes: cell proliferation, DNA repair, and metastasis. The module network, including the KEGG pathways, TFs, miRNAs, and biological processes, is connected according to the three identified IGENs in the three types of bladder cell. By comparing the connection changes of the module networks from normal bladder cells to stage 1 bladder cancer cells, and from stage 1 bladder cancer cells to stage 4 bladder

cancer cells, we ultimately unraveled the cellular mechanisms behind bladder carcinogenesis and proposed two multiple drug combinations for treating stage 1 and stage 4 bladder cancers, respectively.

Additionally, to determine how the two major risk factors, aging and smoking, influence bladder carcinogenesis, we highlighted not only the significantly expressed genes between smokers and nonsmokers, but also the significantly expressed genes between young (≤45 years old) and old (>45 years old) people in the core network biomarkers of bladder carcinogenesis. Finally, we investigated the carcinogenic mechanism of human bladder cells by which the identified major factors, including downregulated miR-1-2, aging, and smoking, lead to the progression from normal bladder cells to stage 1 bladder cancer cells through the SUP and ER signaling pathways. The smoking-related protein HSP90AA1 and DNA methylation of *ECT2* mediate the progression from stage 1 bladder cancer cells to metastasis in stage 4 bladder cancer. Activated DNA repair and accumulated epigenetic alterations lead to the phenotypic changes of bladder cells from normal to cancerous, and from cancerous to metastatic cells owing to the immortality of cancer cells. Based on the core network biomarkers in bladder carcinogenesis, a multiple drug combination comprising gefitinib, estradiol, yohimbine, and fulvestrant was designed for treating stage 1 bladder cancer with minimal side-effects, while a multiple drug combination comprising gefitinib, estradiol, chlorpromazine, and LY294002 was designed for treating stage 4 bladder cancer with minimal side-effects.

2. Materials and Methods

According to the flowchart in Figure 1, we constructed a candidate human IGEN by mining large databases, including BioGRID, TargetScan, HTRIdb, TRANSFAC, and ITFP. However, many false positive and insignificant connections existed in the candidate human IGEN for normal and cancerous bladder cells. Using the NGS expression data, miRNA profiles, and the methylation profiles of normal and cancerous bladder cells in TCGA, we identified the association parameters of the network connections. We also applied AIC to detect the systems order, that is, the number of connections, and to delete the insignificant connections that were out of system order to prune the false positive connections in the candidate IGEN and obtain the two real IGENs for normal and cancerous bladder cells, respectively. By applying PGNP to the two real IGENs in normal and cancerous cells, we first identified the core proteins/TFs that played a major role in the principal networks of the IGENs, constituting the core IGENs in normal and cancerous cells. To determine how the signaling cascades from the core receptor proteins to the core TFs participate in bladder carcinogenesis, the core proteins, which mediate the signal transductions from the core receptor proteins to the core TFs, and their corresponding genes were considered the core network biomarkers of the normal and cancerous cells. The miRNAs with very different connections in regulating the genes of the core network biomarkers between normal and cancerous cells were also involved in the core network biomarkers. Finally, by comparing the connection changes of the core network biomarkers from normal cells to stage 1 cancer cells, and from stage 1 cancer cells to stage 2 cancer cells, we investigated the cellular mechanisms of bladder carcinogenesis.

2.1. Data Preprocessing of Omics Data. We downloaded the genome-wide mRNA and miRNA NGS data and the methylation profiles from TCGA, including 17 samples for normal bladder cells, 348 samples for stage 1 bladder cancer cells, and 56 samples for stage 4, that is, metastatic stage, bladder cancer cells. The data also contained 6 samples for young (≤45 years old) people, 477 samples for old (>45 years old) people, 98 samples for nonsmokers, and 323 samples for smokers. We used one-way analysis of variance (ANOVA) to identify significant differences in gene expression between smokers and nonsmokers, and between young and old people (p value < 0.05). We used the gene symbols of the human gene information data downloaded from the NCBI FTP site as standard human gene names to integrate the omics data, including NGS data, methylation profiles, drug response genome-wide microarray data in CMAP, drug-gene interaction DGIdb data, miRNA-target gene association data in TargetScan, PPIs in BioGRID, transcription regulations in HTRIdb, and ITFP and TRANSFAC data. We also used the GO database, the NCBI Entrez Gene database, and the KEGG pathway database to find the biological processes and pathways of each gene. We used Matlab's text-file and string manipulation tools for text mining.

2.2. Construction of the Stochastic Regression Models for the IGEN System. The goal of the stochastic regression model is to characterize molecular mechanisms, including PPIs, transcription regulations, miRNA regulations, and epigenetic regulations via DNA methylation, by NGS data through detecting false positives of candidate IGENs in human cells. For the stochastic regression model of the gene regulatory subnetwork in the candidate human IGEN, including transcription regulations, miRNA regulations, and epigenetic regulations via DNA methylation, we identified the regulation capabilities of TFs and miRNAs in the GRN of the candidate IGEN. For the expression levels of the ith gene, its DNA methylation and its jth TF/protein and lth miRNA in the nth sample are denoted by $x_i(n)$, $m_i(n)$, $y_j(n)$, and $s_l(n)$, respectively. Then, the stochastic regression model of GRN is described by the following stochastic regression equation:

$$x_i(n) = \sum_{\substack{j \equiv \Omega_i \\ j \neq i}} a_{ij} M_i(n)\, y_j(n) + \sum_{l \equiv \delta_i} c_{li} M_i(n)\, x_i(n)\, s_l(n) + b_i M_i(n) + \nu_i(n), \quad \text{for } i = 1, \ldots, K,\ n = 1, \ldots, N, \tag{1}$$

where the repression ability from the lth miRNA to the ith gene $c_{li} \leq 0$; the basal level of the ith gene expression $b_i \geq 0$; $\Omega_i \subset \Omega \equiv \{1, \ldots, K\}$; $\delta_i \subset \delta \equiv \{1, \ldots, L\}$; $M_i(n) = 1/[1 + (m_i(n)/0.5)^2]$; Ω_i and δ_i denote the candidate regulations based on the databases of transcription regulation and miRNA-target association, respectively; a_{ij} indicates the regulatory ability from the jth TF $y_j(n)$ to the ith gene; $v_i(n)$ represents the stochastic noise due to the modeling residue and fluctuation in the ith gene; and K, L, and N are the total number of TFs, miRNAs, and data samples in the omics data, respectively. $M_i(n)$ denotes the effect of methylation $m_i(n)$ on the binding affinity of TFs, miRNAs, or RNA polymerase on the ith gene which also represents the impact of DNA methylation of the ith gene on the binding affinities of miRNAs, RNA polymerase, and TFs in the gene expression process. The effect on binding affinities $M_i(n)$, for $i = 1, \ldots, K$, ranged between 0.2 and 1, while the expression range of the genome-wide DNA methylation $m_i(n)$, for $i = 1, \ldots, K$, is between 0 and 1. If DNA methylation of the ith gene is close to 1, the effect on the binding affinity to the ith gene is close to 0.2, which implicates the impact of DNA methylation on the binding affinities of miRNAs, RNA polymerase, and TFs to be like an inhibitor. The ith mRNA expression results from transcription regulations $\sum_{j \equiv \Omega_i, j \neq i} a_{ij} M_i(n) y_j(n)$, miRNA repressions $\sum_{l \equiv \delta_i} c_{li} M_i(n) x_i(n) s_l(n)$, the mRNA basal expression $b_i M_i(n)$, and the stochastic noise due to measurement and random fluctuations $v_i(n)$. In model (1), the TF regulations, miRNA regulations, and basal levels are all influenced by the DNA methylation $m_i(n)$ on the ith gene.

For the stochastic regression model of the miRNA regulatory subnetwork in the candidate IGEN, the expression levels of the lth miRNA and its ith target gene in the nth sample, denoted by $s_l(n)$ and $x_i(n)$, respectively, could be described by the stochastic regression model of miRNA regulatory network (MRN) as the following stochastic regression equation:

$$s_l(n) = \sum_{i \equiv \delta_l} c_{li} M_i(n) x_i(n) s_l(n) + M_l(n) z_l + e_l(n), \quad \text{for } l = 1, \ldots, L, \ n = 1, \ldots, N, \tag{2}$$

where the repression ability of the lth miRNA to the ith gene $c_{li} \leq 0$; the basal level of the lth miRNA expression $z_l \geq 0$; $\delta_l \subset \delta \equiv \{1, \ldots, L\}$; δ_l denotes the candidate regulations of the lth miRNA based on the database of miRNA-target gene association; $e_l(n)$ represents the stochastic noise owing to the modeling residue and fluctuation in the lth miRNA. The lth miRNA expression in (2) results from miRNA-gene interactions $\sum_{i \equiv \delta_l} c_{li} M_i(n) x_i(n) s_l(n)$, the miRNA basal expression z_l, and the stochastic noise $e_l(n)$.

For the stochastic regression model of the PPI subnetwork in the candidate IGEN, the expression level of the jth protein and its kth connecting protein in nth sample, denoted by $y_j(n)$ and $y_k(n)$, respectively, could be described by the stochastic regression model of PPIN as the following stochastic regression equation:

$$y_j(n) = \sum_{\substack{k \equiv \Omega_j \\ k \neq j}} d_{jk} y_k(n) y_j(n) + h_j + w_j(n), \quad \text{for } j = 1, \ldots, K, \ n = 1, \ldots, N, \tag{3}$$

where the basal level of the jth protein expression $h_j \geq 0$; $\Omega_j \subset \Omega \equiv \{1, \ldots, K\}$; Ω_j denotes the candidate interactions of the jth protein based on the PPI database; d_{jk} indicates the interaction ability of the kth protein to the jth protein; and $w_j(n)$ represents the stochastic noise owing to the modeling residue and fluctuation in the jth protein. The jth protein expression in (3) results from the rate of formation of the protein complex $y_k(n) y_j(n)$ proportional to the product of the concentration of each protein $\sum_{k \equiv \Omega_j, k \neq j} d_{jk} y_k(n) y_j(n)$ [24, 25], the protein basal expression h_j, and the stochastic noise $w_j(n)$.

We proposed general stochastic regression models to characterize cellular mechanisms, including genetic and epigenetic regulations, in human cells. A number of parameters, including the TF regulatory ability a_{ij}, the miRNA repression ability c_{li}, and the protein interaction ability d_{jk}, needed to be estimated and were determined using the databases of PPI, miRNA-target gene association, and transcription regulation.

2.3. Identification of the TF Regulatory Ability a_{ij}, the miRNA Repression Ability c_{li}, and the Protein Interaction Ability d_{jk} and Their Statistical Significance Testing. We used the mRNA and miRNA expression data from the NGS as the expression levels for $x_i(n)$ and $s_l(n)$, respectively, and used DNA methylation profiles as the expression level of $m_i(n)$ to identify the model parameters a_{ij}, c_{li}, d_{jk}, b_i, z_l, and h_j in (1)–(3). Because large-scale measurement of protein activities has yet to be realized and 73% of the variance in protein abundance can be explained by mRNA abundance [26], mRNA expression profiles were always used to substitute for the protein expression profiles. Therefore, we also applied mRNA expression levels in the NGS data as the expression levels of $y_j(n)$ to identify the parameters in (1)–(3). If the simultaneously measured genome-wide protein expression data and the mRNA expression data in each bladder cancer stage are available, the general models in (1)–(3) can also be applied to identify the real IGEN of the cancer more precisely. Because the parameters in (1) have certain constraints, such as the nonpositive miRNA repressions and nonnegative basal levels, the regulatory parameters were identified by solving the constrained least square parameter estimation problem in the following.

In order to identify the parameters in (1), the stochastic regression model of GRN was rewritten as the following linear regression form:

$$x_i(n) = \left[M_i(n)\, y_1(n) \;\cdots\; M_i(n)\, x_i(n)\, y_K(n) \;\; M_i(n)\, x_i(n)\, s_1(n) \;\cdots\; M_i(n)\, x_i(n)\, s_L(n) \;\; M_i(n)\right],$$

$$\begin{bmatrix} a_{i1} \\ \vdots \\ a_{iK} \\ c_{1i} \\ \vdots \\ c_{Li} \\ b_i \end{bmatrix} + \nu_i(n) = \phi_i(n)\,\theta_i^1 + \nu_i(n), \quad \text{for } i = 1,\ldots,K,\ n = 1,\ldots,N, \tag{4}$$

where $\phi_i(n)$ denotes the regression vector and θ_i^1 is the parameter vector of target gene i to be estimated. $x_i(n)$ and $\phi_i(n)$ are available in the omics data.

The regression model (4) at different data samples can be rearranged as follows:

$$\begin{bmatrix} x_i(1) \\ \vdots \\ x_i(N) \end{bmatrix} = \begin{bmatrix} \phi_i(1) \\ \vdots \\ \phi_i(N) \end{bmatrix} \theta_i^1 + \begin{bmatrix} \nu_i(1) \\ \vdots \\ \nu_i(N) \end{bmatrix}, \tag{5}$$

where N denotes the number of data samples in the NGS data of a bladder cancer stage.

For simplicity, we define the notations X_i, Φ_i, and V_i to represent (5) as follows:

$$X_i = \Phi_i \theta_i^1 + V_i. \tag{6}$$

The constrained least square parameter estimation problem of θ_i^1 is formulated as follows:

$$\min_{\theta_i^1} \quad \left\|\Phi_i\theta_i^1 - X_i\right\|_2^2$$

$$\text{subject to} \quad \begin{bmatrix} \overbrace{0 \;\cdots\; \cdots \;0}^{K} & \overbrace{1 \;0 \;\cdots\; 0}^{L+1} \\ \vdots \;\ddots\; \ddots\; \vdots & 0 \;\ddots\; \ddots\; \vdots \\ \vdots \;\ddots\; \ddots\; \vdots & \vdots \;\ddots\; 1 \;\; 0 \\ 0 \;\cdots\; \cdots \;0 & 0 \;\cdots\; 0 \;-1 \end{bmatrix} \theta_i^1 \tag{7}$$

$$\leq \begin{bmatrix} 0 \\ \vdots \\ \vdots \\ 0 \end{bmatrix}.$$

This gives the constraints to force the miRNA repression c_{li} to be always nonpositive and the basal level b_i to be always nonnegative in (1); that is, $c_{li} \leq 0$ and $b_i \geq 0$. The constrained least square problem was solved using the active set method for quadratic programming [27, 28].

Similarly, the stochastic regression model of the miRNA regulatory subnetwork in (2) was rewritten in the following regression form:

$$s_l(n) = \left[M_1(n)\, x_1(n)\, s_l(n) \;\cdots\; M_K(n)\, x_K(n)\, s_l(n) \;\; M_l(n)\right] \begin{bmatrix} c_{l1} \\ \vdots \\ c_{lK} \\ z_l \end{bmatrix} + e_l(n) = \vartheta_l(n)\,\theta_l^2 + e_l(n), \quad \text{for } i = 1,\ldots,K,\ n = 1,\ldots,N, \tag{8}$$

where $\vartheta_l(t)$ indicates the regression vector and θ_l^2 is the parameter vector to be estimated.

For simplicity, we define the notations S_l, Ψ_l, and E_l to represent (8) as follows:

$$S_l = \Psi_l\theta_l^2 + E_l. \tag{9}$$

The parameter identification problem is then formulated as follows:

$$\min_{\theta_i^2} \quad \left\|\Psi_l\theta_l^2 - S_l\right\|_2^2$$

$$\text{subject to} \quad \begin{bmatrix} \overbrace{1 \;0 \;\cdots}^{K} & 0 \\ 0 \;\ddots\; \ddots & \vdots \\ \vdots \;\ddots\; 1 & 0 \\ 0 \;\cdots\; 0 & -1 \end{bmatrix} \theta_l^2 \leq \begin{bmatrix} 0 \\ \vdots \\ \vdots \\ 0 \end{bmatrix}. \tag{10}$$

This gives the constraint to force the miRNA repression c_{li} to be always nonpositive and the basal level z_l to be always nonnegative in (2); that is, $c_{li} \leq 0$, and $z_l \geq 0$. Finally, the protein model (3) uses the same way like above to make sure $h_j \geq 0$.

Furthermore, in order to extract the core network biomarkers from normal and cancerous cells, we first used NGS data and methylation profiles in the normal and stage 1 and 4 bladder cancer cells to identify an IGEN for normal bladder cells and a general IGEN for bladder cancer cells. The two identified IGENs were used to extract the core network biomarkers in bladder carcinogenesis. We then used the association parameters in the general IGEN of bladder cancer cells as the initial condition of the constrained least square parameter estimation and applied the data on stage 1 and 4 bladder cancer cells to identify the IGENs for stages 1 and stage 4, respectively. According to the three identified IGENs in normal bladder cells, and the stage 1 and 4 bladder cancer cells, we determined the cellular mechanisms of the core network biomarkers in bladder carcinogenesis. The proposed methodology to identify the IGENs for normal bladder cells and stage 1 and 4 bladder cancer cells was summarized in the flowchart in Figure 2.

By applying Student's t-test to the parameter estimation method [29], the p values for the estimated parameters, including the TF regulatory ability a_{ij}, the miRNA repression ability c_{li}, and the protein interaction ability d_{jk}, were calculated to determine the significance of the parameters. Additionally, to determine the significance of expression level and DNA methylation profile of a gene/miRNA between normal bladder cells and cancerous bladder cells, we applied one-way ANOVA to calculate the p value.

After the parameter identification problem had been solved, we identified the IGEN for each bladder cell type. For example, we identified the regulatory parameter $a_{\mathrm{RPS20,JUN}} = 0.26$ from the TF JUN to the target gene RPS20 (p value < 0.02) in stage 4 bladder cancer cells, the interaction parameter $d_{\mathrm{HUWE1,ADRM1}} = 1.2$ between the two proteins ADRM1 and HUWE1 (p value < 0.005) in stage 1 bladder cancer cells, and the coupling rate $c_{\mathrm{RPS20,MIR155}} = -1.2$ between the miRNA miR155 and the mRNA RPS20 in stage 4 bladder cancer cells (p value < 0.07).

2.4. Principal Genome-Wide Network Projection (PGNP). After the identification of the IGENs in normal and cancer cells, we extracted the core network biomarkers of the IGENs based on the perspectives of the functional modules and pathways to reveal the cellular mechanisms behind bladder carcinogenesis. To extract the core network biomarkers, including the core proteins, their corresponding genes, and their upstream miRNAs, from an IGEN on a genome-wide scale, we first decomposed the combined network matrix of the IGEN to left- and right-singular vectors and singular values based on singular value decomposition (SVD). The top left- and right-singular vectors with the top singular values constitute the principal network of the IGEN. The projection distance of each gene/protein/miRNA to these top singular vectors represents the significance of this gene/protein/miRNA in the IGEN. The genes/proteins/miRNAs with the top projection distance ultimately constitute the core network biomarkers of the IGEN. Let the combined network matrix of the TF regulatory ability a_{ij}, the miRNA repression ability c_{li}, and the protein interaction ability d_{jk} of the IGEN in (1)–(3) be represented by

$$A = \begin{bmatrix} a_{11} & \cdots & a_{1K} \\ \vdots & \ddots & \vdots \\ a_{K1} & \cdots & a_{KK} \\ c_{11} & \cdots & c_{1K} \\ \vdots & \ddots & \vdots \\ c_{L1} & \cdots & c_{LK} \\ d_{11} & \cdots & d_{1K} \\ \vdots & \ddots & \vdots \\ d_{K1} & \cdots & d_{KK} \end{bmatrix}. \tag{11}$$

By applying PGP, the matrix A is then be decomposed as follows:

$$\begin{aligned} A &= \mathrm{UDV}^T \\ &= \begin{bmatrix} u_1 & \cdots & u_K \end{bmatrix} \begin{bmatrix} d_1 & 0 & 0 \\ 0 & \ddots & 0 \\ 0 & 0 & d_K \end{bmatrix} \begin{bmatrix} v_1 & \cdots & v_K \end{bmatrix}^T \\ &= \sum_{i=1}^{K} u_i d_i v_i^T, \end{aligned} \tag{12}$$

where $u_i, v_i \in \Re^K$ are the ith left- and right-singular vectors of A, respectively. The diagonal entries of D are the K singular values of A in descending order, $d_1 \geq \cdots \geq d_K$.

The eigenexpression fraction (E_m) is defined as

$$E_m = \frac{d_m^2}{\sum_{m=1}^{K} d_m^2}. \tag{13}$$

We choose the top M singular vectors of V such that $\sum_{m=1}^{M} E_m \geq 0.85$, with the minimal M, so that the top M principal components contain 85% of the IGEN from an energy point of view. The principal projections of A to the top M singular vectors of V, or similarities, are defined as follows:

$$S(k,m) = a_k \cdot v_m^T, \quad \text{for } k = 1, \ldots, (2K+L),\ m = 1, \ldots, M, \tag{14}$$

where a_k and v_m^T denote the kth row vector of A and the mth singular vector of V, respectively. Furthermore, we defined the 2-norm distance from the target genes, miRNAs, and proteins/TFs to the top M singular vectors, respectively, as follows:

$$D(k) = \left[\sum_{m=1}^{M} [S(k,m)]^2 \right]^{1/2}, \quad \text{for } k = 1, \ldots, (2K+L), \tag{15}$$

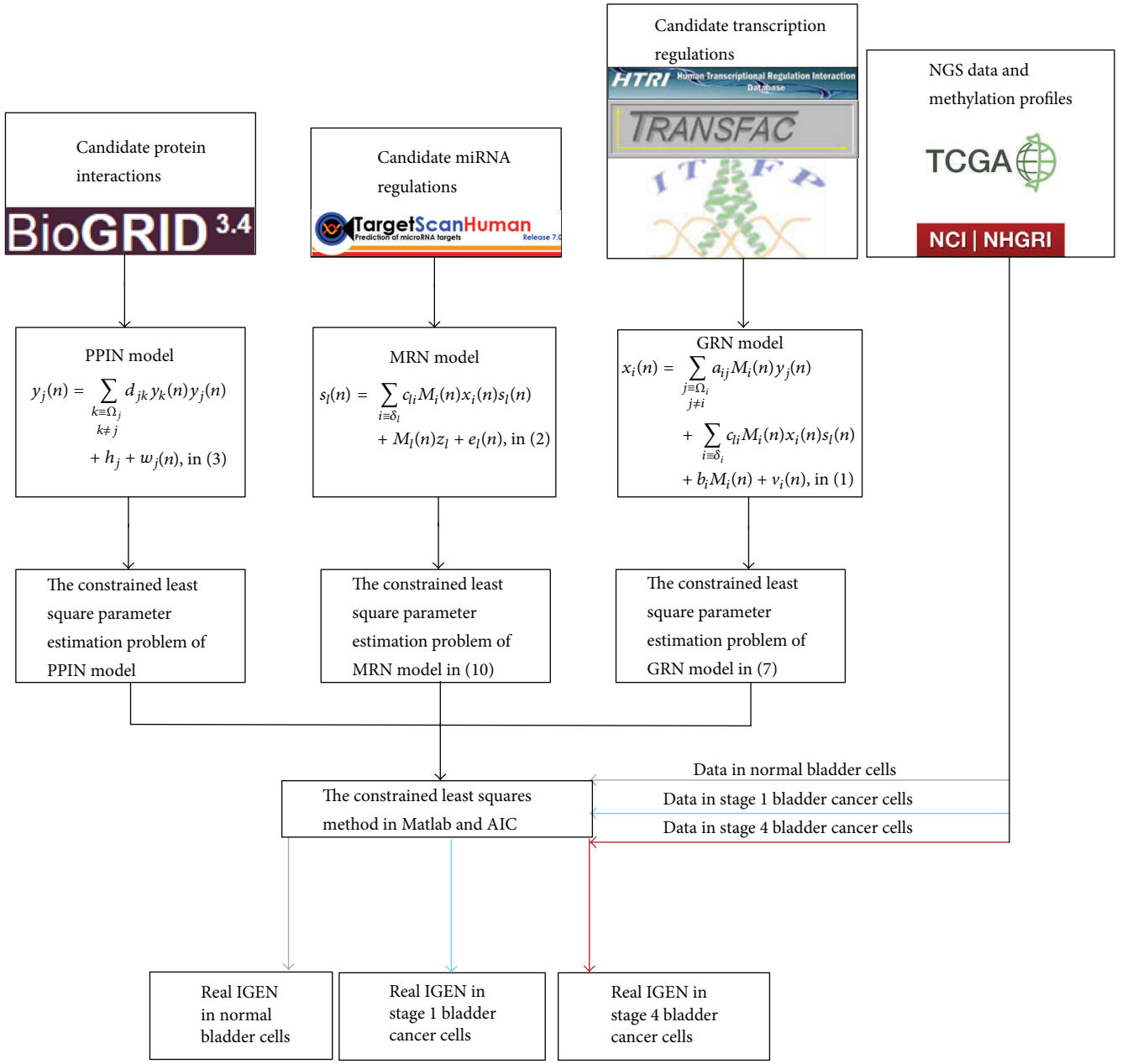


Figure 2: Flowchart of the proposed methodology to identify the IGENs for normal bladder cells, and stage 1 and 4 bladder cancer cells.

where $D(k)$ for $k = 1, \ldots, K$, for $k = K+1, \ldots, K+L$, and for $k = K+L+1, \ldots, 2K+L$ are the 2-norm distances from the target genes, miRNAs, and proteins/TFs to the top M singular vectors, respectively. According to $D(k)$ for $k = K+L+1, \ldots, 2K+L$, we can identify the core proteins/TFs that play a major role in the principal networks of the IGENs, constituting the core IGENs in normal and cancer cells. The identified core proteins/TFs contain receptors that mediate the signaling cascades connected to core TFs. The core proteins, which participate in signal transduction from core receptors to core TFs, and their corresponding genes, were considered the core network biomarkers for normal and cancerous cells. The miRNAs with very different connections in regulating the genes of the core network biomarkers between two cells were also involved in the core network biomarkers.

2.5. Design of a Multiple Drug Combination with Minimal Side-Effects for the Treatment of Bladder Cancer. To design a multiple drug combination with minimal side-effects for the treatment of bladder cancer based on the core network biomarkers of the IGEN, we considered two databases, CMAP and DGIdb. CMAP contains the genome-wide microarray data in response to 1327 drugs in five cell lines, while DGIdb comprises a drug-gene interaction database. Multiple drug therapy induces a genome-wide

response. The strategy of multiple drug screening is that the multiple drugs should inhibit the highly expressed genes, activate the reduced expression of the genes, and not influence the nondifferentially expressed genes in the core network biomarkers of bladder cancer cells compared with normal bladder cells. The binding protein of the designed multiple drug combination can also be obtained using the DGIdb. The strategy leads to improved drug safety and efficacy in the treatment of bladder cancer.

3. Results and Discussion

3.1. Construction of IGEN. We first used NGS expression data and methylation profiles in normal bladder cells and stage 1 and 4 bladder cancer cells to identify a real IGEN for normal bladder cells and a general real IGEN for bladder cancer cells (see Section 2). By applying PGNP to the real IGEN of the normal bladder cells and the general real IGEN of the bladder cancer cells, we then obtained 115 core proteins/TFs for the core IGEN of the normal bladder cells and 138 core proteins/TFs for the core IGEN of the bladder cancer cells. To determine how the signaling cascades from the core receptor proteins to the core TFs participate in bladder carcinogenesis, the core proteins, which mediate the signal transductions from core receptor proteins to core TFs, and their corresponding genes were considered the core network biomarkers. The miRNAs with a high number of different connections regulating the genes of the core network biomarkers between normal and cancerous cells were also involved in the core network biomarkers. Moreover, to identify the mechanism of carcinogenesis from stage 1 to stage 4 bladder cancer, we used the identified parameters of models (1)–(3) in the general IGEN of bladder cancer cells as the initial condition of the constrained least square parameter estimation. We then applied the data for stage 1 and 4 bladder cancer cells to obtain the two real IGENs for stage 1 and 4 bladder cancer, respectively. Furthermore, we analyzed the connection changes of the core network biomarkers between normal bladder cells and stage 1 bladder cancer cells (Figure 3) and between stage 1 and 4 bladder cancer cells (Figure 4) to determine the mechanisms of bladder carcinogenesis and accordingly design multiple drug combinations for treating bladder cancer with minimal side-effects.

To investigate the impact of the major risk factors, aging and smoking, on the core network biomarkers of bladder carcinogenesis, we highlighted the significantly expressed genes between young and old people and between nonsmokers and smokers in the core network biomarkers (p value < 0.05). Additionally, the genes with changes in the basal level of (1) between normal bladder cells and stage 1 bladder cancer cells and between stage 1 and 4 bladder cancer cells were also highlighted in the core network biomarkers of Figures 3 and 4, respectively. The basal level change of a gene between two cell types has been implicated in the epigenetic regulation of gene expression. The expression of a gene that exhibits a basal level change and a significant change (p value < 0.05) of its methylation profile between the two bladder cell types is probably regulated by DNA methylation in bladder carcinogenesis.

3.2. Projection of the Core Network Biomarkers into Biological Processes and Signaling Pathways to Investigate Carcinogenic Mechanisms of Bladder Cancer. According to the information of the biological processes and signaling pathways in the GO and KEGG pathway databases, the roles of the genes in the core network biomarkers (Figures 3 and 4) are projected into three pathways: the SUP, TNF signaling, and ER signaling pathways and three biological processes: cell proliferation, DNA repair, and metastasis.

It has been reported that the SUP pathway is associated with increased proliferation in urinary bladder carcinogenesis [30]. HuaChanSu (HCS), a class of toxic steroids, has been used to show that the TNF pathway mediates the inhibition of cell proliferation in bladder cancer [31]. Moreover, the viability of human bladder cancer cells is reduced by using cantharidin, a natural toxin, through the ER pathway [32]. Therefore, the proteins of the core network biomarkers participating in the SUP, TNF, and ER signaling pathways play an important role in bladder carcinogenesis. We then determined how the core network biomarkers mediate bladder carcinogenesis through the influences of aging, smoking, epigenetic regulation, and miRNA regulation.

The role of the SUP pathway is to degrade misfolded proteins, influence PPIs, translocate proteins, and stabilize protein structure. Owing to the accumulation of genetic mutations and epigenetic alterations in cancer cells, the SUP pathway plays a crucial role in the maintenance of many important cellular processes in cancer cells. The repressed activity of ubiquitin C (UBC), which encodes the polyubiquitin precursor, influences degradation and translation of several proteins in stage 1 and stage 4 bladder cancer cells. For example, the repression of UBC affects the signal transduction of RARRES3, a tumor suppressor, in bladder carcinogenesis. To maintain the cellular functions of cancer cells, the regulation of the SUP pathway adapts to the accumulated genetic mutations and epigenetic alterations.

In normal cells, the TNF pathway is critical for inducing inflammation, which can cause cell death. Accumulated DNA damage, epigenetic alterations, or stresses can induce the TNF pathway, and the pathway then triggers cell death. JUN, one of the TFs in the TNF pathway, plays an important role in promoting the invasion and migration of bladder cancer cells [33]. We determined that the repressed expression of JUN in stage 1 bladder cancer cells leads to cancer cell immortality and causes accumulated genetic mutations and epigenetic alterations. Additionally, the results revealed that JUN was activated in stage 4 bladder cancer cells to mediate metastasis. The role of JUN in the metastasis of bladder cancer cells can also be supported [33]. It has also been reported that the TNF pathway acts as a switch between inflammation and cancer [34]. Moreover, downregulated BCL3, which participates in the TNF pathway in the adipose tissue of the bladder wall, leads to reduced inflammation in bladder carcinogenesis [35].

The ER pathway participates in the regulation of protein folding, protein synthesis, and posttranslational modifications [36]. Misfolded proteins, arising from genetic mutations, epigenetic alterations, or stresses, induce the ER pathway to restore cellular homeostasis in normal cells. Owing to

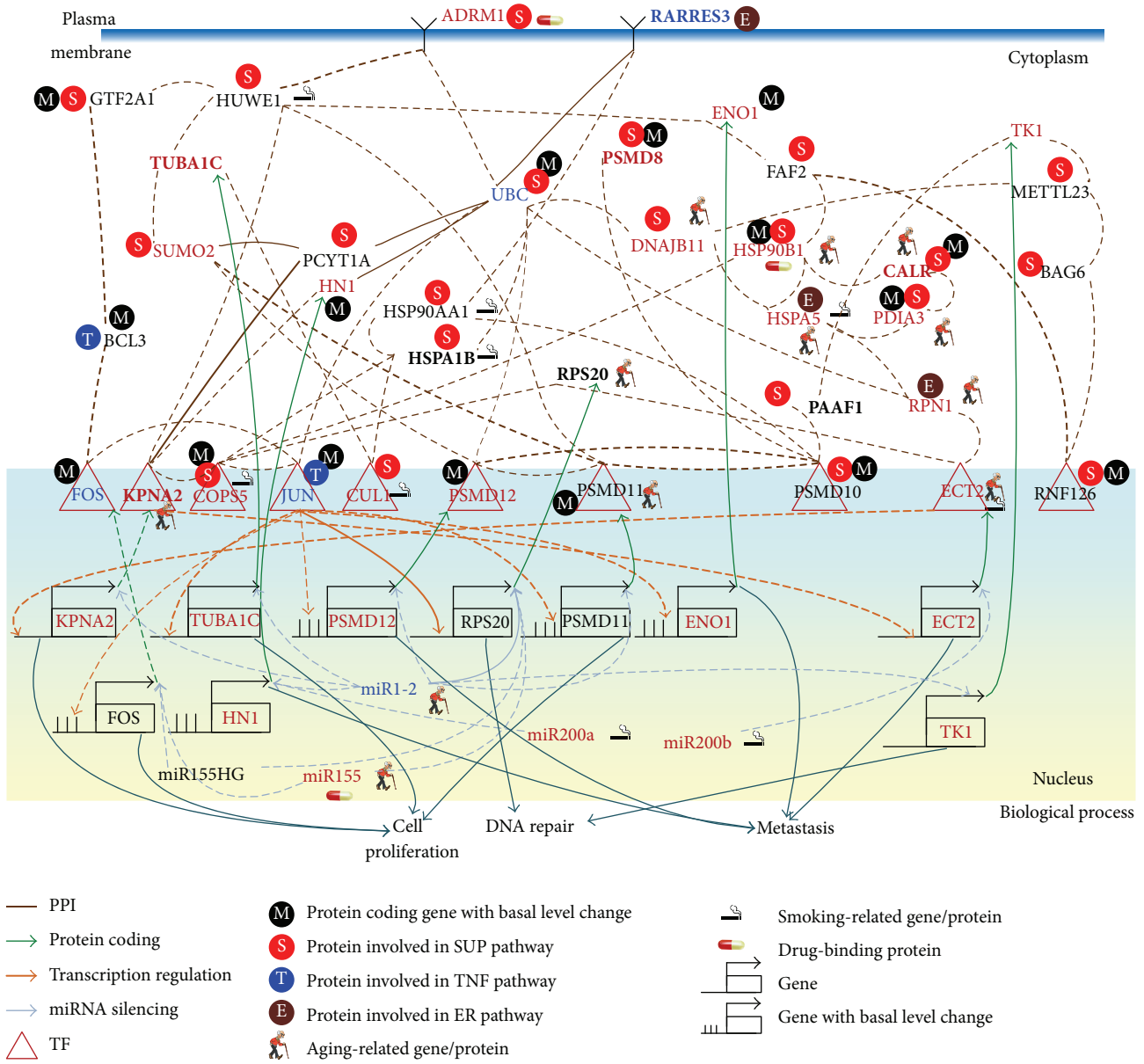


Figure 3: Comparison of genetic and epigenetic alterations and connection changes in the core network biomarkers of bladder carcinogenesis between normal bladder cells and stage 1 bladder cancer cells. Red, blue, and black gene/miRNA symbols represent the highly expressed genes, the suppressed genes, and the nondifferentially expressed genes in stage 1 bladder cancer cells, respectively, compared with normal bladder cells. Dashed and solid lines denote the identified connections in normal and cancerous cells, respectively. The identified connections of the core network biomarkers do not exist in normal bladder cells only. Bold lines indicate the high regulatory or interaction parameters, that is, a_{ij}, c_{li}, and d_{jk}, identified in the stochastic regression models (1)–(3) of the IGEN. The bold proteins, including RARRES3, TUBA1C, PSMD8, HSPA1B, RPS20, CALR, PAAF1, and KPNA2, were the identified core network biomarkers. The major factors, including downregulated miR1-2, the aging-related proteins, HSP90B1, CALR, HSPA5, PDIA3, RPN1, and ECT2, the smoking-related proteins, HUWE1, HSPA5, and ECT2, and the epigenetic regulation of *ENO1*, *HSP90B1*, *CALR*, and *PDIA3*, lead to the progression from normal bladder cells to stage 1 bladder cancer cells through the SUP and ER signaling pathways.

the immortal nature of cancer cells, the accumulated genetic mutations and epigenetic alterations in bladder cancer cells can activate most of the genes that contribute to the ER pathway in bladder carcinogenesis (Figures 3 and 4). In the ER pathway, only RARRES3, a tumor suppressor gene, was downregulated in stage 1 bladder cancer cells.

3.3. The Impact of Aging, Smoking, and miRNA and Epigenetic Regulation on Bladder Carcinogenesis through the Core Network Biomarkers. Major factors, including downregulated miR1-2 and aging- and smoking-related proteins, may lead to the progression from normal bladder cells to stage 1 bladder cancer cells through the SUP and ER signaling pathways.

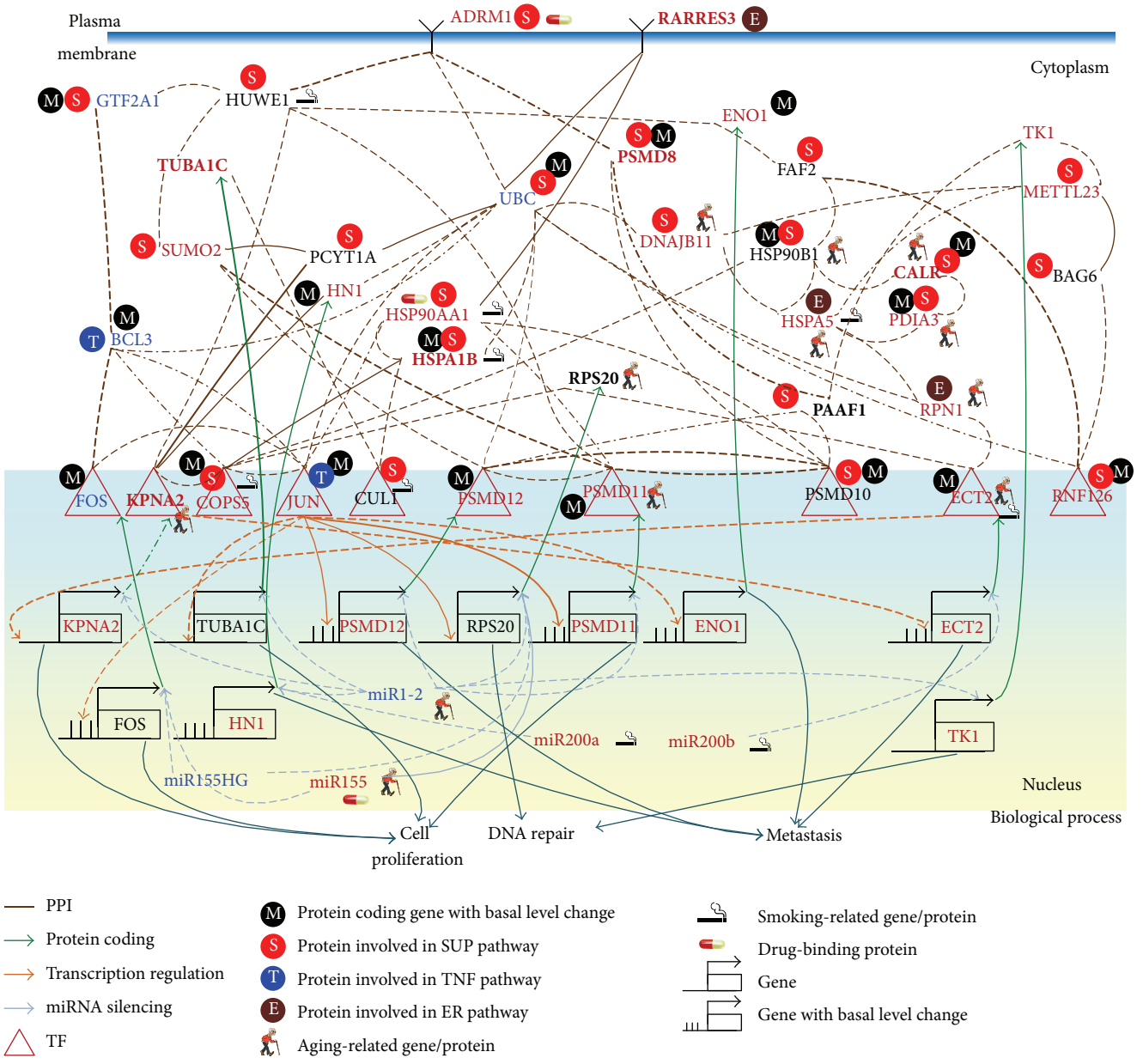


FIGURE 4: Comparison of genetic and epigenetic alterations and connection changes in the core network biomarkers of bladder carcinogenesis between stage 1 and stage 4 bladder cancer cells. Red, blue, and black gene/miRNA symbols represent the highly expressed genes, the suppressed genes, and the nondifferentially expressed genes in stage 4 bladder cancer cells, respectively, compared with stage 1 bladder cancer cells. Dashed, dash-dot, and solid lines denote the identified connections in stage 1 cancer cells, stage 4 cancer cells, and both stage 1 and 4 cancer cells, respectively. Bold lines indicate the high regulatory or interaction parameters, that is, a_{ij}, c_{li}, and d_{jk}, identified in the stochastic regression models (1)–(3) of the IGEN. The bold proteins RARRES3, TUBA1C, PSMD8, HSPA1B, RPS20, CALR, PAAF1, and KPNA2 were the identified core network biomarkers. The smoking-related protein HSP90AA1 and DNA methylation of *ECT2* mediate metastasis of bladder cancer.

It has been reported that aging and smoking are the major factors that accumulate genetic and epigenetic alterations and ultimately induce bladder carcinogenesis. In Figure 3, our results reveal that ADRM1 regulates KPNA2, which promotes proliferation, and is mediated by the aging-related proteins, HSP90B1, CALR, HSPA5, PDIA3, RPN1, and ECT2, the smoking-related proteins, HUWE1, HSPA5, and ECT2, and the epigenetic regulation of ENO1, HSP90B1, CALR, and PDIA3, through the SUP and ER signaling pathways. ADRM1 knockdown leads to a reduction of cancer cell proliferation and has been found in gastric [37], ovarian [38], liver [39], and colorectal cancers [40] and acute leukemia [41]. Therefore, the results support the hypothesis that aging is the most important factor in inducing bladder carcinogenesis through the SUP pathway. Additionally, our results (Figure 3) show that the inhibited aging-related miRNA miR1-2 in

stage 1 bladder cancer cells leads to miR1-2 dysregulation of genes including *KPNA2*, *TUBA1C*, *HN1*, *PSMD11*, *PSMD12*, and *TK1*, which influence cell proliferation, DNA repair, and metastasis. miR1-2 has also been identified as a tumor suppressor in bladder cancer cells [42].

3.4. miR1-2 and miR200b Mediate the Reduction of Cell Proliferation and Metastasis through KPNA2 and ECT2, Respectively. The receptor ADRM1 signal triggers the signaling cascade from the smoking-related protein HUWE1 to the aging-related proteins HSP90B1 and RPS20 and the smoking-related TF COPS5. The TF COPS5 upregulates the metastasis-associated gene *ECT2*, which is suppressed by miR200b in stage 1 bladder cancer cells. The results show the cross-regulation between the transcription of the smoking-related protein COPS5 and the aging-related protein ECT2. The aging-related miRNA miR1-2 and the smoking-related miRNA miR200b act as a switch to depress the proliferation-associated protein KPNA2 in stage 1 and stage 4 bladder cancer cells (Figures 3 and 4) and the metastasis-associated gene *ECT2* in stage 4 bladder cancer cells (Figure 4), respectively.

3.5. The Smoking-Related Protein HSP90AA1 and DNA Methylation of ECT2 Mediate the Metastasis of Bladder Cancer. Our results reveal that receptor RARRES3 signaling triggers the activated TF JUN mediated by the smoking-related protein HSP90AA1, and JUN then activates the metastasis-associated gene *PSMD12* in stage 4 bladder cancer cells (Figure 4). Receptor ADRM1 signaling also triggers the metastasis-associated protein PSMD12 through the proteins PSMD8 and PAAF1 and epigenetic regulation in stage 4 bladder cancer cells. This shows that metastasis-associated *ECT2* is activated by epigenetic regulation in stage 4 bladder cancer cells. Receptor RARRES3 signaling also triggers the aging-related and proliferation-associated TF PSMD11 through the smoking-related protein HSP90AA1 in stage 4 bladder cancer cells. The activated TF JUN also regulates the proliferation-associated gene *PSMD11* and the DNA repair-associated gene *RPS20*. There is evidence that curcumin (diferuloylmethane) can suppress tumor initiation, promotion, and metastasis. Curcumin can also inhibit the expression of JUN [43]. Additionally, the RNAi-induced induction of *ECT2* suppresses cell migration, invasion, and metastasis [44]. Our results indicate that the upregulation of *ECT2* in stage 4 bladder cancer cells is regulated by epigenetic regulation of *ECT2* expression. This is also supported by the significant change in the DNA methylation profiles in *ECT2* between normal bladder cells and stage 4 bladder cancer cells (p value < 0.007).

3.6. Functional Module Network Analysis in Bladder Carcinogenesis. The activated DNA repair of bladder cancer cells leads to metastasis owing to the immortality of cancer cells.

According to the modular information in the GO database and the KEGG pathway database, the genes/proteins in the core network biomarkers (Figures 5 and 6) are projected into three pathways, the SUP pathway, the TNF signaling pathway, and the ER signaling pathway, and three biological processes: cell proliferation, DNA repair, and metastasis. The module networks in Figures 5 and 6 show that the activated TFs KPNA2, COPS5, PSMD12, and ECT2 play an important role in mediating the signal transduction of the SUP and ER pathways to activate cell proliferation and metastasis in stage 1 bladder cancer. The metastasis of the stage 1 bladder cancer is repressed by the activated miRNAs miR200a and miR200b, as shown in Figure 5. The activated signal transduction from SUP and ER pathways also triggers DNA repair through the epigenetically regulated TFs PSMD11 and RNF126.

Additionally, the activated TFs PSMD11, RNF126, and JUN mediate the signal transduction from SUP, TNF, and ER pathways to trigger cell proliferation, DNA repair, and metastasis in stage 4 bladder cancer, as shown in Figure 6. Although miR155 is activated in stage 1/4 bladder cancer, miR155 suppresses FOS and RPS20 in stage 1 bladder cancer, and miR155 only suppresses the DNA repair-associated gene *RPS20* in stage 4 bladder cancer. Furthermore, we suggest that DNA repair may play a critical role in repairing DNA damage, which results from genetic and epigenetic alterations, leading to phenotypic change of the bladder cells from normal cells to stage 1 cancer cells, and from stage 1 cancer cells to metastatic cancer cells.

In summary, aging and epigenetic regulation dominate bladder carcinogenesis through CALR, PDIA3, DNAJB11, HSPA5, RPN1, HSP90B1, KPNA2, ECT2, and PSMD11 and through COPS5, PSMD8, RNF126, CALR, PDIA3, HSP90B1, PSMD12, PSMD11, JUN, HN1, and ENO1, respectively. Smoking promotes bladder carcinogenesis especially in metastasis. Finally, the cellular mechanisms from normal to stage 1 bladder cancer cells and from stage 1 to stage 4 bladder cancer cells are summarized in Figures 7(a) and 7(b), respectively. When the accumulated genetic mutations and epigenetic alterations lead to the dysregulation of the TNF pathway in inflammation, the accumulated misfolded proteins in the ER pathway induce cell proliferation in stage 1 bladder cancer (Figure 7(a)). Regulation of the ER and TNF pathways adapts to the accumulated genetic mutations and epigenetic alterations through the SUP pathway. The progression of DNA repair and cell proliferation in stage 1 bladder cancer ultimately results not only in the repression of miR200a and miR200b during metastasis, but also in the regulation of the TNF pathway to metastasis, cell proliferation, and DNA repair in stage 4 bladder cancer (Figure 7(b)).

3.7. Two Separate Drug Combinations for Treating Stage 1 and Stage 4 Bladder Cancer Cells with Minimal Side-Effects. The design of a multiple drug combination for treating stage 1 bladder cancer depends on a strategy of inhibiting the highly expressed genes *ADRM1*, *COPS5*, *PSMD8*, *SUMO2*, *CALR*, *PDIA3*, *DNAJB11*, *HSPA5*, *RPN1*, *CUL1*, *HSP90B1*, *KPNA2*, *PSMD12*, *ECT2*, *TK1*, *TUBA1C*, *HN1*, and *ENO1*; activating the suppressed genes *UBC*, *JUN*, *RARRES3*, and *FOS*; and suppressing the drug's effect on the nondifferentially expressed genes *BAG6*, *HUWE1*, *PAAF1*, *PSMD10*, *FAF2*, *PCYT1A*, and *PSMD10*. According to the drug design strategy (see Section 2), a multiple drug combination comprising gefitinib, estradiol, yohimbine, and fulvestrant was obtained for treating stage 1 bladder cancer.

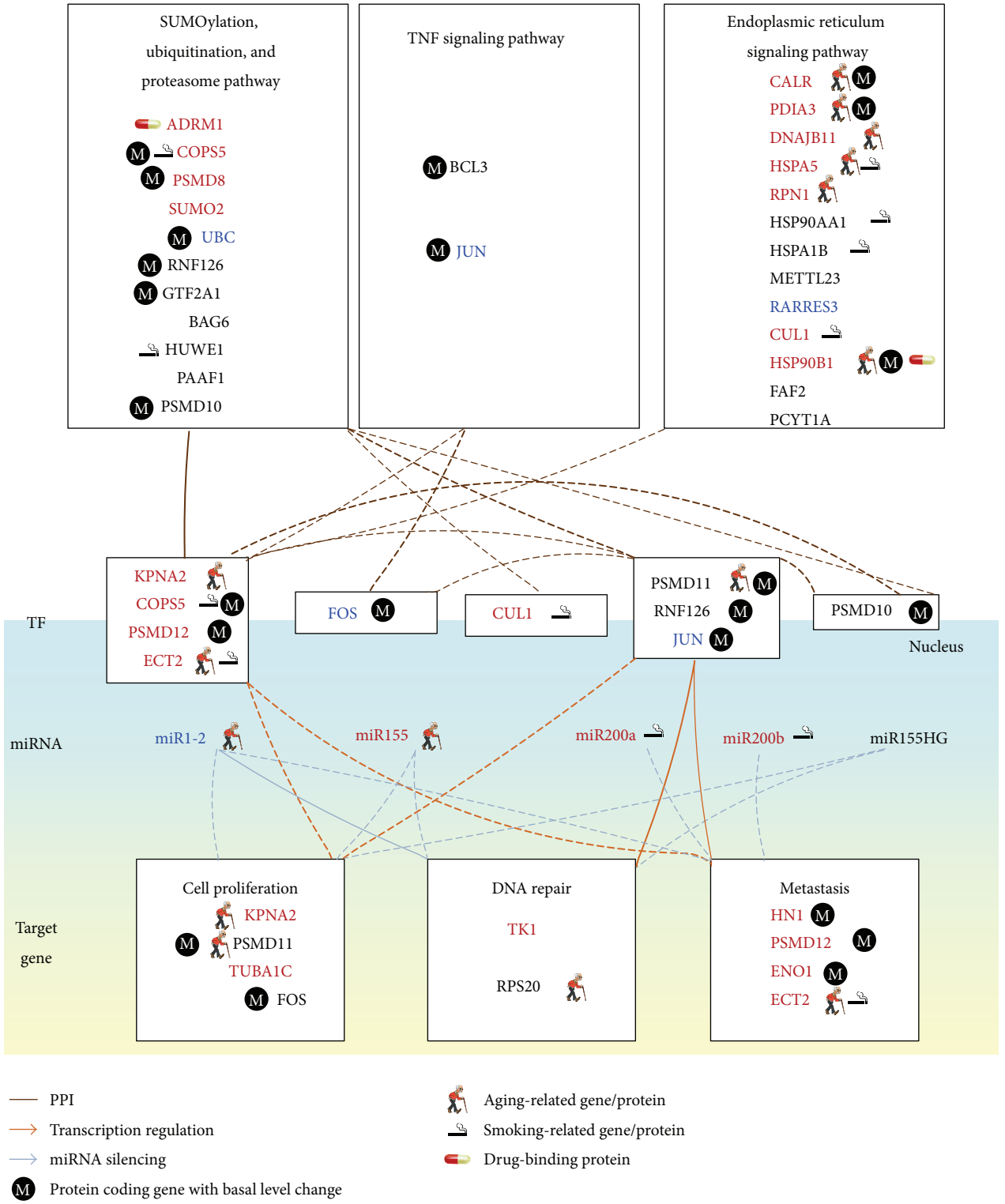


Figure 5: Module network of the core network biomarkers in Figure 3 for investigating the bladder carcinogenic mechanisms from normal bladder cells to stage 1 bladder cancer cells. The notations of gene/miRNA symbols and line styles are the same as those in Figure 3. The activated TFs KPNA2, COPS5, PSMD12, and ECT2 play an important role in mediating the signal transduction of the SUP and ER pathways to activate cell proliferation and metastasis in stage 1 bladder cancer. The metastasis of the stage 1 bladder cancer is repressed by the activated miRNAs miR200a and miR200b.

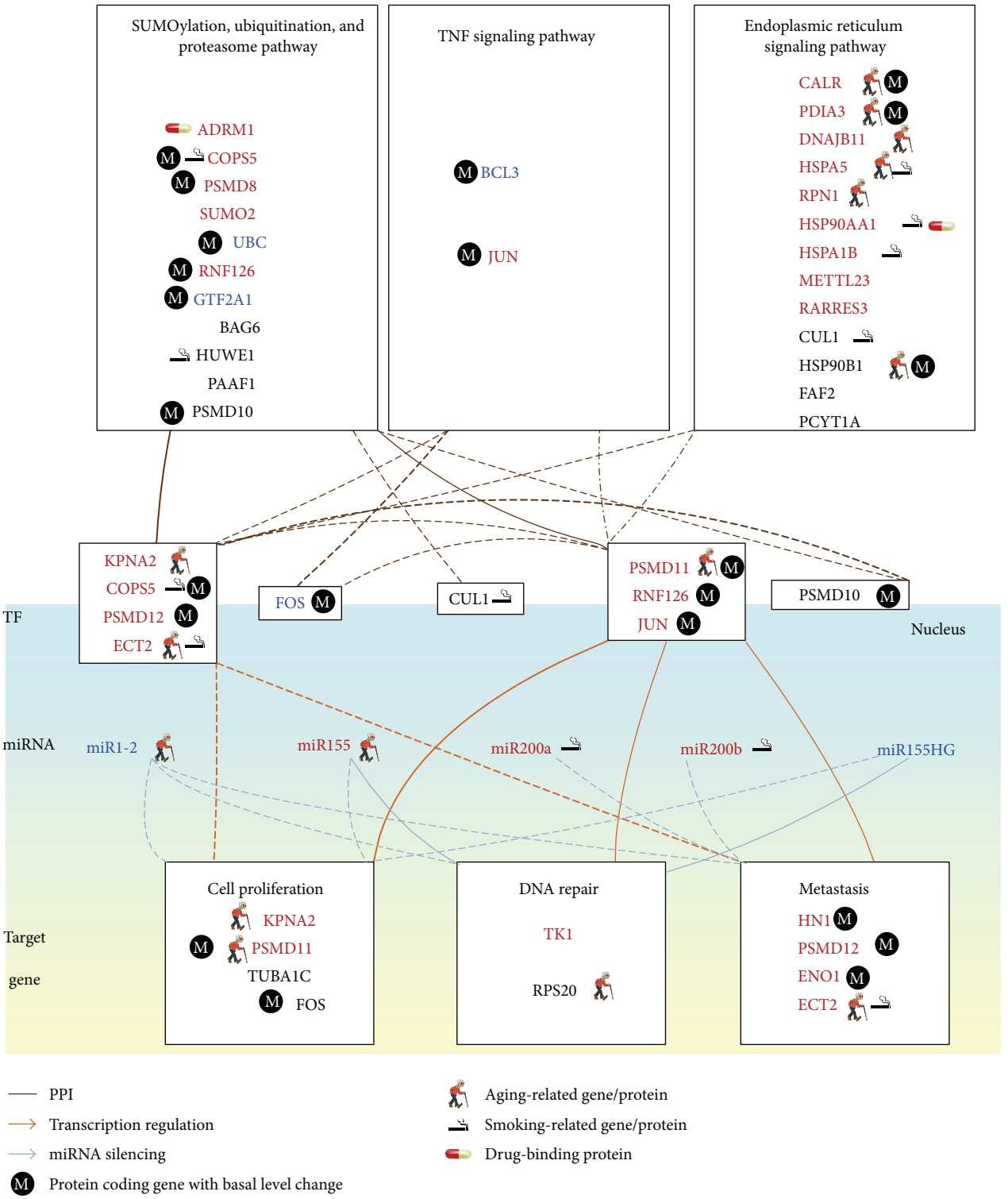
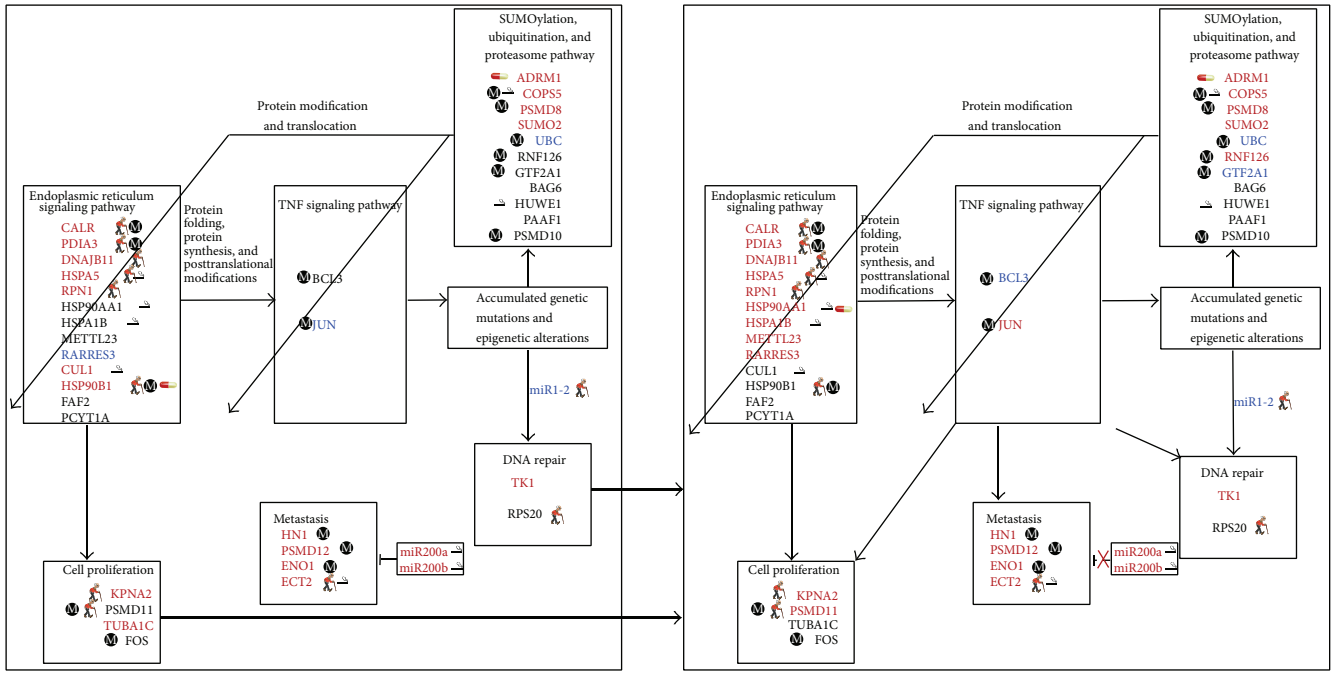


Figure 6: Module network of the core network biomarkers in Figure 4 to investigate the bladder carcinogenic mechanisms from stage 1 to stage 4 bladder cancer cells. The notations of gene/miRNA symbols and line styles are the same as those in Figure 4. The activated DNA repair of bladder cancer cells leads to metastasis owing to the immortality of cancer cells. The activated JUN in the TNF pathway induces cell proliferation, DNA repair, and metastasis in stage 4 bladder cancer cells.



(a) Carcinogenic mechanism from normal to stage 1 bladder cancer

(b) Carcinogenic mechanism from stage 1 to stage 4 bladder cancer

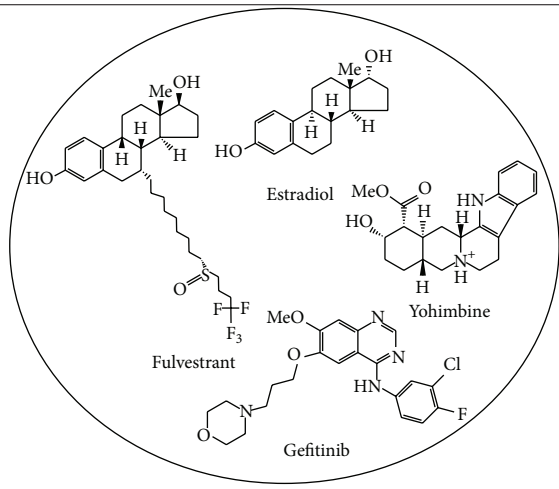
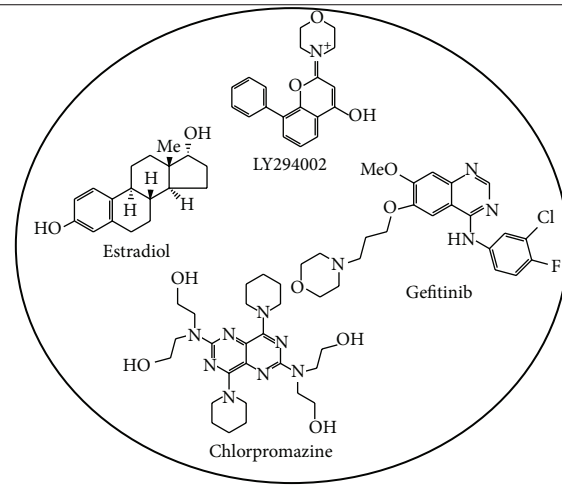
FIGURE 7: The carcinogenic mechanisms from normal to stage 1 bladder cancer cells (a), and from stage 1 to stage 4 bladder cancer cells (b). When the accumulated genetic mutations and epigenetic alterations lead to the dysregulation of the TNF pathway in inflammation, the accumulated misfolded proteins in the ER pathway induce cell proliferation in stage 1 bladder cancer (a). The regulations of ER and TNF pathways are adaptive to the accumulated genetic mutations and epigenetic alterations through the SUP pathway. The progression of DNA repair and cell proliferation in stage 1 bladder cancer ultimately results not only in the repression of miR200a and miR200b during metastasis, but also in the regulation of the TNF pathway to metastasis, cell proliferation, and DNA repair in stage 4 bladder cancer (b).

The design of a multiple drug combination for treating stage 4 bladder cancer depends on a strategy of inhibiting the highly expressed genes *ADRM1*, *COPS5*, *PSMD8*, *SUMO2*, *RNF126*, *CALR*, *PDIA3*, *DNAJB11*, *HSPA5*, *RPN1*, *HSP90AA1*, *HSPA1B*, *METTL23*, *RARRES3*, *KPNA2*, *PSMD12*, *ECT2*, *JUN*, *TK1*, *TUBA1C*, *HN1*, and *ENO1*; activating the suppressed genes *BCL3*, *FOS*, *UBC*, and *GTF2A1*; and suppressing the drug's effect on the nondifferentially expressed genes, which are the same as those in stage 1 bladder cancer. We obtained a multiple drug combination comprising gefitinib, estradiol, chlorpromazine, and LY294002 for treating stage 4 bladder cancer. According to the information in DGIdb, miR-155, the HSP90 protein family, ADRM1, and estrogen receptor are the direct targets of the multiple drug combination comprising gefitinib, estradiol, yohimbine, and fulvestrant in stage 1 bladder cancer, respectively (Figures 3 and 5), while the same proteins are also the direct targets of the multiple drug combination comprising gefitinib, estradiol, chlorpromazine, and LY294002 in stage 4 bladder cancer, respectively (Figures 4 and 6). Moreover, the analysis of drug response genome-wide microarray data reveals that high doses of yohimbine can activate BAG6 in stage 1 bladder cancer, while high doses of chlorpromazine can activate HSPA5 and JUN in stage 4 bladder cancer. Therefore, low-dose yohimbine and low-dose chlorpromazine could avoid side-effects in the treatment of stage 1 and stage 4 bladder cancer cells, respectively. Ultimately, we designed one specific drug combination for treating stage 1 bladder cancer and another specific drug combination for treating stage 4 bladder cancer with minimal side-effects (Table 1).

4. Conclusion

In this study, we proposed a new method for constructing an IGEN for characterizing cellular mechanisms in bladder carcinogenesis by using system regression modeling and large-scale database mining. We then applied PGP to obtain the core network biomarkers of the IGEN. By comparing the connection changes of the core network biomarkers between normal bladder cells and stage 1 bladder cancer cells and between stage 1 and stage 4 bladder cancer cells, we investigated the progression mechanisms of bladder carcinogenesis. Database mining provided all possible candidates for genetic and miRNA regulations and protein interactions in IGEN. We used AIC and statistical assessment to prune the false positive regulations and interactions by applying the regression coupling model to NGS data and methylation profiles. We compared the connection differences in the core network biomarkers between different cellular types to explore bladder carcinogenic mechanisms. According to the comparison of the connection changes in the core network biomarkers between normal cells and stage 1 cancer cells

Table 1: The multiple drug design strategy and potential multiple drug combination for stage 1 and 4 cancers.

	Stage 1 bladder cancer	Stage 4 bladder cancer
The highly expressed genes for potential inhibition strategy of multiple drug design	*ADRM1*, *COPS5*, *PSMD8*, *SUMO2*, *CALR*, *PDIA3*, *DNAJB11*, *HSPA5*, *RPN1*, *CUL1*, *HSP90B1*, *KPNA2*, *PSMD12*, *ECT2*, *TK1*, *TUBA1C*, *HN1*, and *ENO1*	*ADRM1*, *COPS5*, *PSMD8*, *SUMO2*, *RNF126*, *CALR*, *PDIA3*, *DNAJB11*, *HSPA5*, *RPN1*, *HSP90AA1*, *HSPA1B*, *METTL23*, *RARRES3*, *KPNA2*, *PSMD12*, *ECT2*, *JUN*, *TK1*, *TUBA1C*, *HN1*, and *ENO1*
The suppressed genes for potential activation strategy of multiple drug design	*UBC*, *JUN*, *RARRES3*, and *FOS*	*BCL3*, *FOS*, *UBC*, and *GTF2A1*
The nondifferentially expressed genes to avoid side-effect of multiple drug design	*BAG6*, *HUWE1*, *PAAF1*, *PSMD10*, *FAF2*, *PCYT1A*, and *PSMD10*	*BAG6*, *HUWE1*, *PAAF1*, *PSMD10*, *FAF2*, *PCYT1A*, and *PSMD10*
The potential multiple drug combination		

and between stage 1 and stage 4 cancer cells, we investigated how the genetic and epigenetic regulations, miRNA regulations, and aging-related and smoking-related genes affect the biological functions that lead to bladder carcinogenesis. According to gene expression changes in the core network biomarkers between normal bladder cells and stage 1 bladder cancer cells and between stage 1 and stage 4 bladder cancer cells, we then identified two separate drug combinations for treating stage 1 and 4 bladder cancer cells. Therefore, the proposed IGEN construction method and PGP provide potential network biomarkers for bladder cancer diagnosis and treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Molecular Biomarkers in Bladder Cancer: Novel Potential Indicators of Prognosis and Treatment Outcomes

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Although many clinical and molecular markers for predicting outcomes in bladder cancer (BC) have been reported, their application in clinical practice remains unclear. Bladder carcinogenesis has two distinct molecular pathways that direct the development of BC. *FGFR3* mutations are common in low-grade BC, while *TP53* mutation or loss of *RB1* is associated with muscle-invasive BC. However, no tissue-based gene markers confirmed by prospective large-scale trials in BC have been used in clinical practice. Micro-RNA analyses of BC tissue revealed that miR-145 and miR-29c* function as tumor suppressors, whereas miR-183 and miR-17-5p function as oncogenic miRNAs. In liquid biopsy, circulating tumor cells (CTC), exosomes, or cell-free RNA is extracted from the peripheral blood samples of cancer patients to analyze cancer prognosis. It was reported that detection of CTC was associated with poor prognostic factors. However, application of liquid biopsy in BC treatment is yet to be explored. Although several cell-free RNAs, such as miR-497 in plasma or miR-214 in urine, could be promising novel circulating biomarkers, they are used only for diagnosing BC as the case that now stands. Here, we discuss the application of novel biomarkers in evaluating and measuring BC outcomes.

1. Introduction

Bladder cancer is the most common cancer of the urinary tract, with an incidence rate of 350,000~380,000 cases being reported per year worldwide [1, 2]. Its main histological type is urothelial carcinoma. There are two clinical phenotypes of bladder cancer: (1) non-muscle- invasive bladder cancer and (2) muscle-invasive bladder cancer. Non-muscle-invasive cancers frequently recur at approximate rate of 50~70% and progress to muscle-invasive disease at rate of 1~2% and ~45% in low-grade and high-grade tumors, respectively [3–5]. Muscle-invasive cancers have a 5-year survival rate of <50% [6, 7]. As it stands now, treatment for muscle-invasive bladder cancer is chiefly total cystectomy. Therapeutic methods against advanced bladder cancer are chemotherapies that mainly use cisplatin as the first-line treatment and have still not made significant progress for more than 20 years.

A set of clinical and pathological parameters are used for risk stratification in bladder cancer, such as number of tumors, size of the tumor, prior recurrence rate, T-stage, presence of carcinoma *in situ*, tumor grade, lymph node status, and variant histology. However, they are only limited to predicting clinical outcomes [8]. Novel therapies for advanced muscle-invasive bladder cancer and established predictive biomarkers on response to them are yet to be identified. Hence, predictive biomarkers for the development of target therapy and novel therapies for advanced bladder cancer must be identified.

Here, we discuss the application of molecular predictive biomarkers in advanced muscle-invasive bladder cancer as well as molecular markers of poor prognosis in postcystectomy patients. Moreover, we discuss the current findings of liquid biopsy in patients with advanced bladder cancer as

well as those of histopathological analyses of primary bladder cancer.

2. Gene Abnormalities in Primary Bladder Cancer

Some theories on the putative molecular mechanism underlying bladder cancer have been demonstrated. Firstly, it is important to understand the molecular pathway of carcinogenesis in bladder cancer before exploring prognostic and predictive molecular markers of advanced bladder cancer. The concept of "field cancerization" was introduced in 1953, which is the theory of multicentric cancer origins [9]. A population of cells in morphologically normal epithelium possessed common genetic or epigenetic aberrations, similar to that observed in bladder cancer, which might provide a ground for multiple tumorigenesis [10]. On the other hand, the "clonal origin" theory states that bladder tumors arise from the uncontrolled spread of a single transformed cell that can grow independently with variable subsequent genetic alterations [11]. Since recent molecular biological approach showed various samples from metachronous and synchroallopatric tumors could arise from monoclonal origin by analyses according to the pattern of X-chromosome inactivation, *TP53* mutation, and loss of heterozygosity, "clonal origin" theory is currently supported on bladder cancer [11–13]. Then, a family of genes has been characterized that follows this "two-hit" model including the two prototype suppressor genes: the retinoblastoma 1 (*RB1*) and *TP53* genes [14]. It is now well established that accumulation of genetic alterations forms the basis for progression from a normal cell to a cancer cell, referred to as the process of multistep carcinogenesis [15, 16]. Recent analyses demonstrated that non-muscle-invasive and muscle-invasive bladder cancers have distinct pathways in carcinogenesis. One pathway involves mutation of *FGF receptor 3*, thereby giving rise to low-grade non-muscle-invasive papillary tumors that frequently recur but seldom invade. In contrast, muscle-invasive bladder cancer and carcinoma *in situ* exhibit deletions or mutations of the *TP53*, *RB1*, *ERBB2*, or *PTEN* [16].

Originally, the *HRAS* gene was the first human oncogene identified from a human bladder cancer cell line. A point mutation alters the 12th amino acid of the *HRAS* oncogene product p21 [17]. It was reported that HRAS was frequently overexpressed in non-muscle-invasive cancer [18]. However, recent reports showed that the *RAS* genes mutations are present in only 1–13% of bladder cancer and less frequent in muscle-invasive cancer [19–21]. Moreover, the *RAS* gene mutation was not a predictor for disease-specific survival [22].

FGFR3 (fibroblast growth factor receptor 3), a receptor tyrosine kinase, is one of the most frequently mutated genes in bladder cancer. The rate of mutation in non-muscle-invasive bladder cancer is 60–70% [23–25]. FGFR3 plays a critical role in bladder cancer from low-grade stage [26], which is characterized by low levels of protein synthesis and high cell cycle gene activity [27]. However, its mutation is comparatively less common in patients with muscle-invasive bladder cancer at the time of diagnosis and has not been established to be as a prognostic biomarker in advanced bladder cancer.

TP53, a transcription factor, has many functions, such as induction of apoptosis, inhibition of cell proliferation, and arrest of the cell cycle. Nuclear accumulation of TP53 is a predicting factor of poor prognosis in advanced bladder cancer [28, 29]. In multivariable analyses of 692 patients with invasive cancer treated with radical cystectomy and lymphadenectomy, TP53 expression was independently associated with disease recurrence and cancer-specific mortality [30]. However, the authors mentioned that assessing TP53 expression has limited utility in patients with lymph node-positive bladder cancer. In addition, the *TP53* gene alteration, which is a poor prognostic factor, is found in 53% of patients who underwent cystectomy [31].

The *RB1*, a tumor suppressor gene, is a negative regulator of the cell cycle, and its alterations are related to carcinogenesis in several cancers. Loss of RB1 expression is an adverse prognostic biomarker in muscle-invasive bladder cancer [32]. Bladder cancer with mutation of the *RB1* gene exhibits low FGFR3 levels and is associated with significantly poor disease-specific survival [33]. The *TSC1* is a tumor suppressor gene, located on chromosome 9q34. TSC1 is a negative regulator of mTOR signaling in complex with TSC2. Deletions of the long arm of chromosome 9 are the most common genetic alteration in bladder cancer. TSC1, and TSC2, which constitute the mTOR regulatory tuberous sclerosis complex, are mutated at a combined frequency of 15% [34]. Thus, everolimus might have activity in metastatic urothelial cancer patients who harbor the *TSC1* mutation. The *TSC1* mutation possibly has a causative role in the progression and this process is possibly related to the functional loss of p27 [35].

Other genes, such as the *ERBB2/HER2* [36, 37] and *PTEN* [38], have been reported to be involved in the progression of advanced bladder cancer. Thus, the signaling pathway from receptors of tyrosine kinase to AKT/PI3K would definitely play a role in carcinogenesis and development of bladder cancer and other cancers. However, only analysis of promising single gene predictive biomarker in bladder cancer has limitations to apply to clinical practice. Further prospective evaluation would be required.

3. MicroRNA Expression in Primary Bladder Cancer

MicroRNAs (miRNAs) are 18–24-nucleotide-long noncoding RNA that inhibit gene function by endogenous blocking. Several miRNAs are involved in carcinogenesis as tumor suppressor or oncogenic molecules.

miR-145, miR-143, and miR-125b are tumor suppressors that are downregulated in bladder cancer tissue, whereas miR-183, miR-96, miR-17-5p, and miR-20a are oncogenic miRNAs that are upregulated in bladder cancer tissue [39]. miR-145 is one of the most recurrently downregulated miRNAs in bladder cancer. miR-141 and miR-205 are poor prognostic biomarkers of overall survival in bladder cancer [40]. miR-29c* expression is severely decreased in advanced cancer. Non-muscle-invasive bladder cancer (50%) with low miR-29c* expression subsequently progressed, whereas 94%

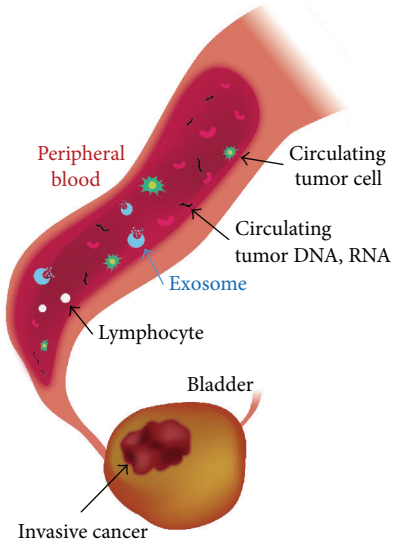

Figure 1: Schema of liquid biopsy.

of non-muscle-invasive bladder cancers with high expression did not progress [41].

4. "Liquid Biopsy" with Patients' Blood Samples: Circulating Tumor Cells, Exosomes, and miRNAs (Figure 1)

Advanced technology uses patients' blood or urine as samples instead of primary bladder cancer tissue to analyze bladder cancer prognosis and to explore novel prognostic or predictive biomarkers. Liquid biopsy involves the analyses of circulating tumor cells (CTCs), exosomes, and circulating miRNAs in patients' blood or urine in human cancers [42]. In the CORRECT analysis for advanced colorectal cancer, a high concordance was observed between plasma circulating DNA and tumor tissue for the *KRAS* and *PIK3CA* mutations [43]. We have been able to select the most effective drug to treat patients with advanced prostate cancer by the CTC assay, using patients' peripheral blood samples. Detection of AR-V7 (Androgen-Receptor splice Variant 7 messenger RNA) in CTCs from patients with castration-resistant prostate cancer may be associated with resistance to enzalutamide and abiraterone [44].

The CTC assay has a potential role in the management of bladder cancer. Next-generation sequencing analysis showed somatic variant detection in 50% of patients with neoadjuvant bladder cancer [45]. At an early stage of diagnosis for bladder cancer, quantitation of CTC from blood and urine by FRα (folate receptor α) ligand-targeted polymerase chain reaction (PCR) could be a promising method for diagnosis [46]. In addition, it was reported that detection of CTC was associated with poor prognostic factors. CTCs were detected in 20% of patients with high-risk non-muscle-invasive bladder cancer and effectively predicted both time-to-recurrence and progression-free survival [47]. Another study showed that the presence of CTC in patients with metastatic bladder cancer was associated with poor survival. However, there was no difference in survival between the CTC-positive and CTC-negative patients with localized bladder cancer [48]. Preoperative CTCs in peripheral blood are detected in 23% of non-metastatic advanced bladder cancer. There was concordance between HER2 expression on CTC and the *HER2* gene amplification status of the primary tumor and lymph node metastases in CTC-positive cases [49]. Their studies had limitations in the small sample size.

Exosomes are small (30–100 nm) membrane vesicles released into the extracellular environment on fusion of multivesicular bodies with the plasma membrane [50]. Exosomes play several physiological roles, such as immune response modulation, presentation of antigens to immune cells, intercellular communication through transfer of proteins, mRNA, and miRNA. However, no *in vivo* research on exosomes has been reported for bladder cancer. Exosomes isolated from the urine of patients with muscle-invasive bladder cancer induced epithelial-to-mesenchymal transition in urothelial cells [51]. A new insight into the role of exosomes in transition of bladder cancer into muscle-invasive cancer was provided. Thus, exosome research in advanced bladder cancer could be a new platform for predicting progression and innovating targeted therapy.

miRNAs are found within cells and in serum and other body fluids. The function of these extracellular circulating miRNAs is not well understood. Extracellular miRNAs embedded in circulating exosomes serve as prognostic biomarkers in cancer. A number of plasma exosomal RNAs are reported as being diagnostic, prognostic, or even therapeutic biomarkers in cancer patients [52]. Circulating exosomal RNAs contain various RNA species and changes in exosomal RNA contents are robust candidates as clinical biomarkers for advanced prostate cancer [53, 54]. Circulating miR-497 and miR-663b in plasma were expressed with significant difference in bladder cancer [55]. Although these miRNAs could be promising novel circulating biomarkers, they are used for only diagnosing bladder cancer as the case now stands. Thus, circulating miRNAs in blood or urine could be biomarkers at several clinical stages, such as detection of bladder cancer, prediction of transition to muscle-invasive disease, or prediction of outcomes. However, urinary cell-free miR-214 or miR-155 has been reported to be used only as a diagnostic biomarker [56, 57], and few studies have reported the relationship between circulating miRNAs and bladder cancer.

In the near future, liquid biopsy detecting CTCs, exosomes, and miRNAs will serve as a tool to predict outcomes and to select effective targeted therapies. Although circulating cells and DNA analyses would be promising because of their convenient and minimally invasive procedures, they are limited by their sample collection methods, lack of sensitivity and specificity, or high costs.

5. Conclusions

Despite the impressive development of recent research on CTC, microRNA, and exosomes, our knowledge pertaining to the biology of bladder cancer lags behind that pertaining to other solid cancers. Therapeutic methods against advanced

bladder cancer have still not made significant progress for more than 20 years, since chemotherapies mainly use cisplatin as the first-line treatment of choice for advanced bladder cancer. One of the reasons for this could be the dramatically different mechanisms underlying carcinogenesis in non-muscle-invasive bladder cancer and muscle-invasive high-grade cancer, which could complicate molecular research on bladder cancer. Establishment of molecular profiling from the context of large clinical trials is required to stratify patients before treatment with conventional chemotherapy. However, currently available data suggest that single biomarkers are inadequate for the surveillance of high-risk patients. Owing to this new angle, combinations of considerably different approaches, such as the use of epigenetic and genetic biomarkers, primary tissue and CTC samples, and genomics and proteomics, are being proposed for successful validation of a robust prediction tool for bladder cancer. Moreover, novel effective agents with the ability to increase the overall survival rate of bladder cancer need to be identified for clinical use.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Urinary APE1/Ref-1: A Potential Bladder Cancer Biomarker

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Bladder cancer (BCa) is one of the most common urothelial cancers with still noticeable incidence rate. Early detection of BCa is highly correlated with successful therapeutic outcomes. We previously showed that apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) was expressed at an increased level in the serum of BCa patients when compared to the level in healthy controls. In this study, we investigated whether urinary APE1/Ref-1 was also elevated in patients with BCa. In this case-control study, voided urine was collected from 277 subjects including 169 BCa patients and 108 non-BCa controls. Urinary APE1/Ref-1 level was assessed by enzyme-linked immunosorbent assay (ELISA). APE1/Ref-1 levels were significantly elevated in BCa patients relative to levels in non-BCa controls and were correlated with tumor grade and stage. Urinary APE1/Ref-1 levels were also higher in patients with recurrence history of BCa. The receiver operating characteristics (ROC) curve of APE1/Ref-1 showed an area under the curve of 0.83, indicating the reliability and validity of this biomarker. The optimal combination of sensitivity and specificity was determined to be 82% and 80% at a cut-off value of 0.376 ng/100 μL for detection of APE1/Ref-1 in urine. In conclusion, urinary APE1/Ref-1 levels measured from noninvasively obtained body fluids would be clinically applicable for diagnosis of BCa.

1. Introduction

Bladder cancer (BCa) is the second most common of all genitourinary malignancies in the United States [1] and Korea [2]. Most individuals with BCa who are diagnosed early, show no muscle invasion, and have superficial urothelial carcinoma can expect a 5-year survival rate of more than 90% [3]. If the BCa is invasive, however, with tumor cells spreading beyond the bladder to the surrounding tissue or to nearby lymph nodes, or organs, signs of late stage BCa, the 5-year survival rate drops sharply. Therefore, early intervention can dramatically increase the probability of a BCa patient's survival. Patients with noninvasive BCa frequently show a high rate of recurrence and progression of the disease within 2 years of transurethral resection [4], and continuous follow-up testing is required. Several studies have focused on the development of tools for the diagnosis and prognosis of BCa using urinary biomarkers [5–9].

In our previous report, we proposed a new BCa diagnostic protein, apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) in serum [10]. APE1/Ref-1 protein was originally identified as a multifunctional protein involved in DNA repair and redox signaling. APE1/Ref-1 levels were found to be elevated with dysregulated cellular proliferation, as is typically seen in cancers [10–12]. APE1/Ref-1 is mainly localized in the nucleus and shows dynamic shuttling between the nucleus and cytoplasm in response to various stress stimuli. Furthermore, extracellular secretion of APE1/Ref-1 into the circulation suggests this protein could be used as a serologic biomarker [13, 14]. APE1/Ref-1 secretion from cells is also supported by the presence of autoantibody against APE1/Ref-1 in the blood of patients with lung cancer [15] and systemic lupus erythematosus [16].

The clinical need for specific and sensitive urothelial tumor diagnostics remains as urgent issue. The ideal diagnostics would measure the level of a BCa protein biomarker

Table 1: Clinicopathological characteristics of patients with bladder cancer.

	Control	Bladder cancer		Total
		NMIBC	MIBC	
N	108	157	12	169
Age (mean ± SEM)	60.9 ± 16.3	68.4 ± 10.4	66.0 ± 9.3	68.23
Sex *N* (%)				
Male	62 (57.4)	133 (84.7)	10 (83.3)	205 (74.0)
Female	46 (42.6)	24 (15.3)	2 (16.7)	72 (26.0)
Smoking history	58 (48.3)	98 (62.4)	8 (66.7)	106 (62.7)
Median tumor size (cm)	n.a.	1.7	3.7	2.4
Number of tumor multiplicities	n.a.	86 (55.8)	10 (66.7)	96 (56.8)
Tumor stage	n.a.			
Ta		108 (68.8)		108 (63.9)
T1		49 (31.2)		49 (17.7)
T2			10 (83.3)	10 (3.6)
T3-4			2 (16.7)	2 (0.7)
Tumor grade	n.a.			
Low		98 (62.4)	0 (0.0)	98 (58.0)
High		59 (37.6)	12 (100.0)	71 (42.0)
No recurrence	n.a.	90 (57.3)	8 (66.7)	98 (58.0)
Previous recurrence		67 (42.7)	4 (33.3)	71 (42.0)

n.a.: nonapplicable; NMIBC: non-muscle-invasive bladder cancer; MIBC: muscle-invasive bladder cancer. Numbers in parentheses are percentages.

in a single step with a noninvasive sampling method. The protein components of urine are qualitatively similar to those of blood although they are more diluted [17]. A diagnostic based on patient urine has some advantages as urine is available in large quantities and can be obtained by noninvasive means for repeated measurements, continuous surveillance, or establishing of assay reproducibility.

In this study, we compared the urinary APE1/Ref-1 levels of patients with BCa and healthy subjects. Using an existing, quantitative APE1/Ref-1 serum assay that was modified for use with urine sample, we aimed to quantify the level of APE1/Ref-1 in urine samples from patients with results from cystoscopic examination of the bladder to establish a new, noninvasive urinary BCa biomarker.

2. Materials and Methods

2.1. Patients and Urine Samples. Urine samples were obtained from 277 patients. All analyses were performed within 6 months of collection. The study subjects were classified as either noncancer controls ($n = 108$), individuals with no evidence of malignancy, or patients with bladder tumors ($n = 169$). Control patients were diagnosed as having nonmalignant urological diseases including benign prostate hyperplasia ($n = 37$), neurogenic bladder ($n = 23$), urolithiasis ($n = 21$), stress urinary incontinence ($n = 19$), and urethral stricture ($n = 8$). Tumors were staged according to the 2011 tumor-node-metastasis (TNM) classification [18] and graded according to the World Health Organization (WHO) system [19] by two pathologists that were blind to this study. In the BCa group, postoperative histological confirmation of urothelial cell carcinoma, including grade and stage, was recorded. All patients' clinical pathological data, including prior medical history and treatment, recurrence, and clinical stage, were retrieved from electronic medical records (Table 1). Voided urine samples were obtained from patients with BCa before cystoscopy and then frozen in liquid nitrogen and stored at −70°C until use. All samples were obtained from the archives of the Department of Urology, Chungnam National University, from 2011 to 2012. The collection and analysis of all samples were approved by the Chungnam National University Hospital institutional review board, and informed consent was obtained from all study subjects.

2.2. Urine Sample Preparation. Each urine sample was thawed and sedimented by centrifugation (10,000 ×g for 10 min) to remove impurities. The supernatant was carefully collected and prepared without an additional protein enrichment step.

2.3. Measurement of Urinary APE1/Ref-1. The level of APE1/Ref-1 in each urine sample was quantitatively analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [10], with some modification. Briefly, 96-microwell plates (Thermo Fisher Scientific, Waltham, MA, USA) were precoated overnight with 100 μL of a 1 : 5000 dilution of a rabbit anti-APE1/Ref-1 antibody (Abcam, Cambridge, UK) in coating buffer (0.5 M carbonate buffer, pH 9.6) in each well. Plates were washed five times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) between all incubation steps. After blocking with blocking buffer (5% bovine serum albumin in PBS-T) at room temperature for 2 h, plates were washed five times with PBS-T. Urine sample, standards, and blank (100 μL/well) were added to the wells. Plates were incubated at 37°C for 90 min and

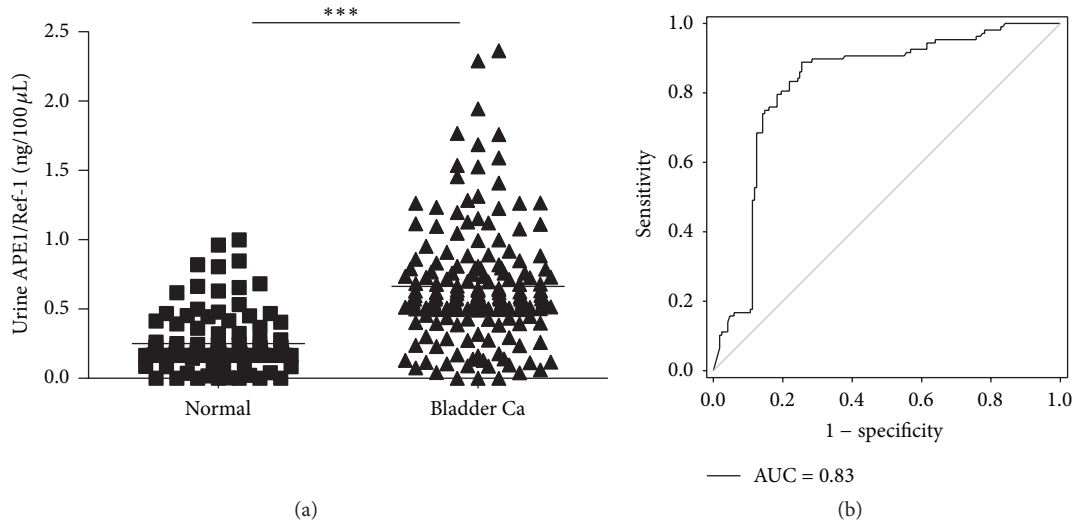

Figure 1: Urinary APE1/Ref-1 levels are elevated in bladder cancer. (a) Urine APE1/Ref-1 levels were measured using an enzyme-linked immunosorbent assay (ELISA). The results are presented as a scatter plot. Each dot represents one patient (n = 108 for noncancer controls; n = 169 for bladder cancer patients). (b) Receiver operating characteristics (ROC) curves of APE1/Ref-1 in bladder cancer detection. The area under the curve (AUC) for the detection of all cancers by APE1/Ref-1 was 0.826.

then washed five times with PBS-T to ensure the removal of remnants that could affect antibody recognition. This was followed by the addition of 100 μL of a 1 : 5000 dilution of a mouse anti-APE1/Ref-1 antibody (Abcam, Cambridge, UK) and further incubation at room temperature for 2 h. The plates were then washed seven times with PBS-T, and 100 μL of horseradish peroxidase-conjugated anti-mouse antibody (1 : 5000) was added; the plate was incubated at room temperature for 30 min. After washing, 100 μL of freshly prepared tetramethyl benzidine substrate was added to the wells. The color development reaction was stopped by adding 100 μL of 2.5 M H_2SO_4, and the absorbance was measured at 450 nm with an automatic microtiter plate reader (Sunrise Xfluor4; Tecan Systems, Inc., San Jose, CA, USA). Each sample was assayed in duplicate, and mean values were determined. A 6-point standard curve was established using purified recombinant human APE1/Ref-1 (rh APE1/Ref-1) [10]. The protein (1 μg/mL) was serially diluted (5-fold) in diluent and used at 0.0064–4 ng/100 μL.

2.4. Statistical Analysis. Data were expressed as the mean ± standard error of the mean (SEM). A repeated-measures ANOVA was used to compare more than three variables, while Student's t-test was used to evaluate differences between two variables. Receiver operating characteristic (ROC) curves were generated by plotting the sensitivity value against the false-positive rate (1 − specificity). We assessed the predictive value of APE1/Ref-1 for BCa by calculating the area under the curve (AUC), and we estimated the optimal cut-off value (Youden index), based on maximum sensitivity and specificity [20]. SPSS version 18.0 for Windows was used to analyze the data. Differences were considered statistically significant if the null hypothesis could be rejected with >95% confidence ($p < 0.05$).

3. Results

3.1. Urine Levels of Human APE1/Ref-1 in Patients with Bladder Cancer. To determine whether urine APE1/Ref-1 levels are elevated in patients with BCa, we used a sandwich ELISA for human APE1/Ref-1 with rh APE1/Ref-1 protein as a standard. Urine levels of APE1/Ref-1 in patients with BCa were significantly higher than those in the control subjects (0.663 ± 0.032 ng/100 μL (n = 169) for BCa patients, 0.250 ± 0.020 ng/100 μL (n = 108) for the control group, $p < 0.01$ (Figure 1(a))). ROC analysis of control versus BCa groups (Figure 1(b)) yielded an AUC of 0.83; the ROC curve and corresponding AUC show that urine APE1/Ref-1 as a biomarker has the predictive ability to discriminate between patients with BCa and normal subjects. The optimal cut-off value was set to maximize the sum of sensitivity and specificity. Based on these results, the optimal combination of sensitivity (81.7%) and specificity (79.6%) was obtained at a cut-off value of 0.3765 ng/100 μL (Table 2).

3.2. Urine APE1/Ref-1 Levels Are Associated with Bladder Tumor Grade, Stage, and Recurrence. The association between levels of APE1/Ref-1 in urine and tumor grade, stage, invasion status, and recurrence were investigated to determine whether APE1/Ref-1 levels are predictive of BCa diagnosis. As shown in Figure 2(a), significant differences in median urinary APE1/Ref-1 levels were found between control (n = 108) and low-grade tumor groups (0.498 ± 0.035 ng/100 μL, n = 98; $p < 0.01$) and between control and high-grade tumor groups (0.982 ± 0.048 ng/100 μL, n = 71; $p < 0.01$). The ROC AUC for low-grade and high-grade BCa were 0.73 and 0.95, respectively (Figure 2(b)). These results suggest that the increased urinary APE1/Ref-1 levels correlate with severity of BCa disease and, therefore, can help guide treatment. We also determined the association

Table 2: Receiver operating curve analysis of APE1/Ref-1 values in patients with bladder cancer.

Cut-off (ng/100 μL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
.2820	84.0	75.9	84.5	75.2
.2900	83.4	75.9	84.4	74.5
.2970	82.8	75.9	84.3	73.9
.3085	82.2	75.9	84.2	73.1
.3195	81.7	75.9	84.7	72.6
.3230	81.7	76.9	84.7	72.8
.3430	81.7	77.8	85.2	73.0
.3650	81.7	78.7	85.7	73.3
.3765	81.7	79.6	86.3	73.5
.3855	81.1	79.6	86.2	72.9
.3895	80.5	79.6	86.1	72.3
.3935	80.5	80.6	86.6	72.5
.3955	79.9	80.6	86.5	71.9
.3975	79.3	80.6	86.5	71.3
.4005	78.7	80.6	86.4	70.7
.4040	78.1	80.6	86.3	70.2
.4095	78.1	81.5	86.8	70.4
.4145	78.1	82.4	87.4	70.6
.4200	78.1	83.3	88.0	70.9

PPV: positive predictive value; NPV: negative predictive value.

of urinary APE1/Ref-1 levels and BCa stage and depth of tumor invasion into the bladder wall. The urinary APE1/Ref-1 levels were significantly elevated in patients with T1 (0.622 ± 0.043 ng/100 μL), T2 (0.657 ± 0.049 ng/100 μL), and T3-T4 (1.065 ± 0.048 ng/100 μL) stage BCa, compared to levels in subjects of the control group (Figure 2(c)), indicating that urinary APE1/Ref-1 level may discriminate between tumor stages. The ROC AUC for T1, T2, and T3-T4 stages were 0.79, 0.85, and 0.99, respectively (Figure 2(d)). In addition, the median urinary APE1/Ref-1 level in patients with non-muscle-invasive BCa (NMIBC; 0.633 ± 0.033 ng/100 μL, n = 157) was significantly lower than that in patients with muscle-invasive bladder cancer (MIBC; 1.065 ± 0.048 ng/100 μL, n = 12, $p < 0.01$; Figure 2(e)). The ROC AUC for NMIBC and MIBC were 0.81 and 0.99, respectively (Figure 2(f)). Both BCa groups had significantly higher levels than the controls (control versus NMIBC $p < 0.01$; control versus MIBC $p < 0.01$). As shown in Table 2, 71 patients (42%) had history of recurrence in the 169 patients with bladder cancer. Urinary APE1/Ref-1 levels in patients with previously recurrent BCa (0.803 ± 0.057 ng/100 μL) were significantly higher than in patients without recurrence (0.563 ± 0.034 ng/100 μL, $p < 0.01$; Figure 2(g)). In ROC analysis, the AUC for patients without recurrent BCa was 0.78, and the AUC for patients with previously recurrent BCa was 0.88 (Figure 2(h)).

3.3. Urine Levels of APE1/Ref-1 in Patients with Hematuria. False-positive results in the nuclear matrix protein 22 (NMP22) test have been observed in cases with hematuria [21]. Therefore, we investigated the effect of hematuria on urine APE1/Ref-1 levels in human subjects. As shown in Figure 3, urine APE1/Ref-1 levels in controls with microscopic or gross hematuria were not significantly different from levels in controls without hematuria (0.270 ± 0.031 ng/100 μL, controls with hematuria, $n = 30$; 0.243 ± 0.025 ng/100 μL, control without hematuria, $n = 78$, $p = 0.596$). Urine APE1/Ref-1 levels of controls with hematuria (0.270 ± 0.031 ng/mL, $n = 30$) were significantly lower than those of BCa group (0.663 ± 0.032 ng/100 μL, $n = 169$, $p < 0.01$). These results show that urine APE1/Ref-1 levels were not affected by microscopic and gross hematuria.

4. Discussion

In the present study, to measure urinary APE1/Ref-1 levels, we modified an APE1/Ref-1 ELISA that was initially developed for the detection of serum APE1/Ref-1 [10]. We obtained an ROC curve by applying results of human urine samples testing and determined a Youden index-based, optimal cut-point for the urinary diagnostic test [20]. A cut-off APE1/Ref-1 value of 0.3765 ng/100 μL resulted in a sensitivity of 81.7% and a specificity of 79.6% of the assay. We also observed that APE1/Ref-1 protein levels were elevated in BCa patients, even those with low-grade tumors, compared with the levels in normal controls. Furthermore, the increased urinary APE1/Ref-1 levels were associated with BCa severity, suggesting that this protein could be used as a reliable marker for BCa diagnosis.

We previously reported serum APE1/Ref-1 as a BCa biomarker [10]. However, to date, there are still no blood-based biomarker tests for use in diagnosis or surveillance of BCa in clinical practice although markers in blood are useful in determining prognosis and informing therapeutic decisions. However, particularly for monitoring bladder diseases, biomarkers in urine have distinct advantages, including larger

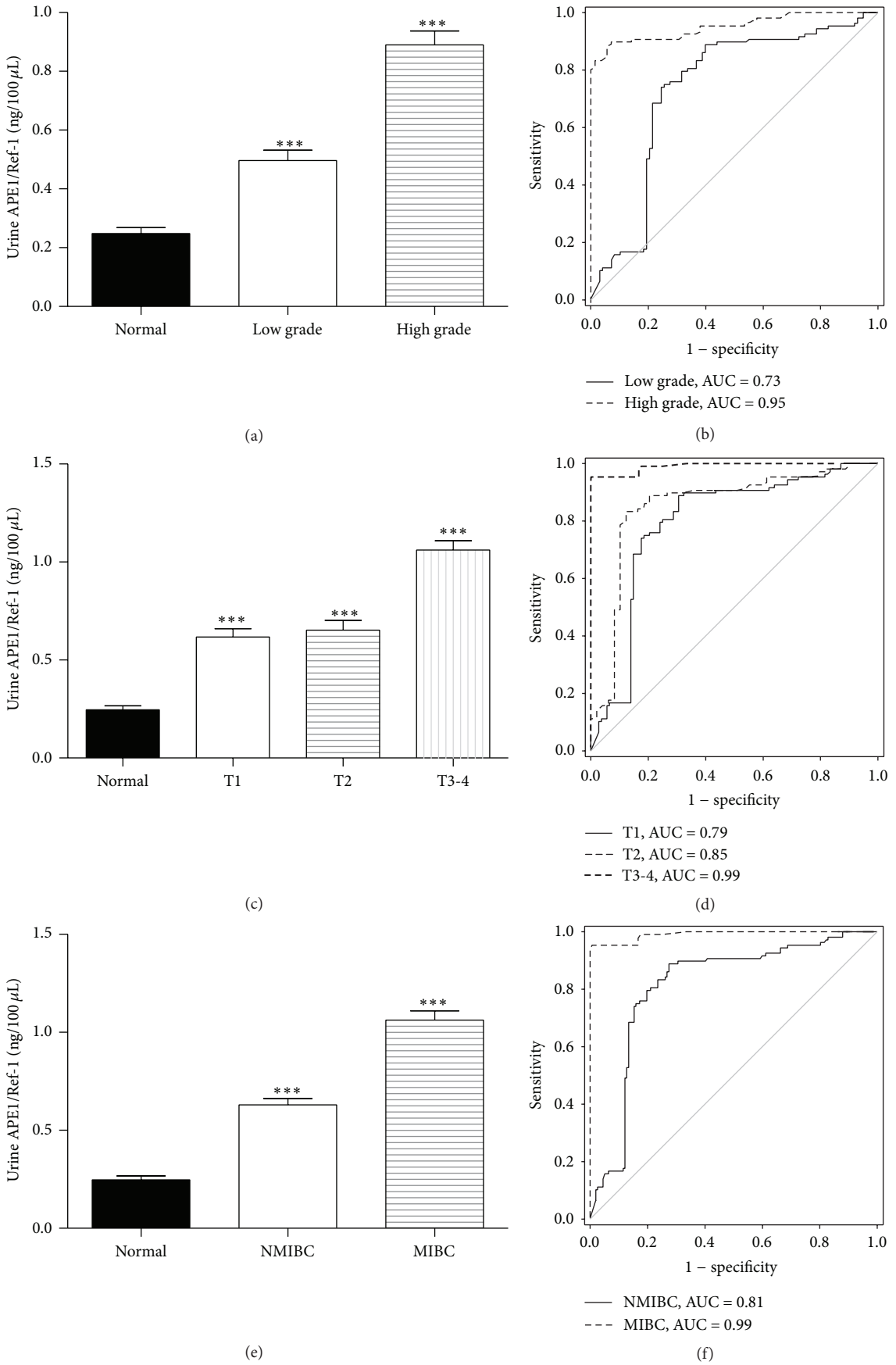

Figure 2: Continued.

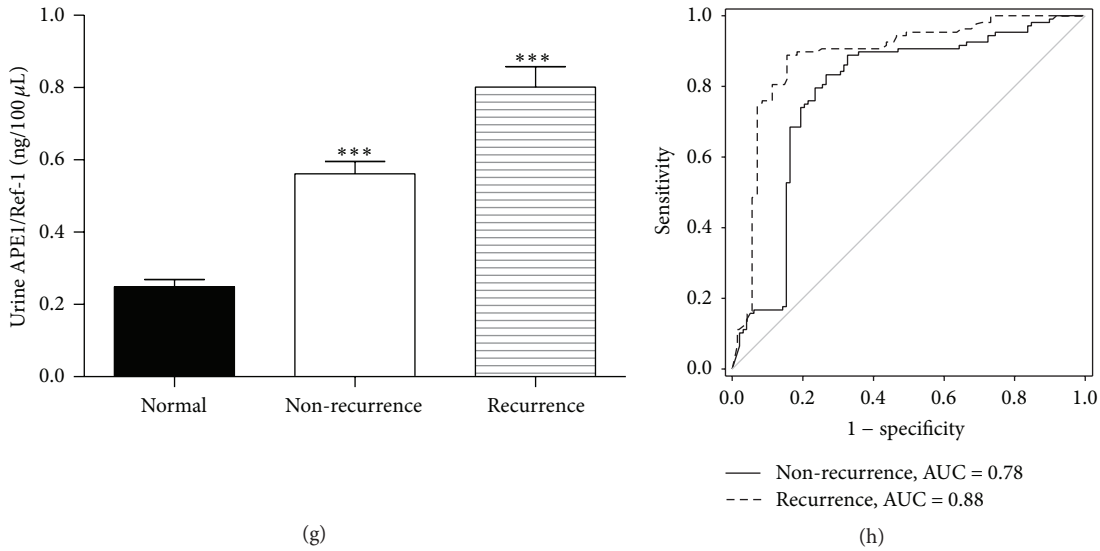

Figure 2: Urinary APE1/Ref-1 levels are associated with bladder tumor grade, stage, muscle invasion, and recurrence. (a) Each bar shows the mean ± standard error of the mean (SEM) (n = 98 for low-grade tumors, n = 71 for high-grade tumors, and n = 108 for noncancer controls). (b) ROC curves for APE1/Ref-1, the determination of different bladder tumor grades. (c) Urinary APE1/Ref-1 levels are elevated in patients with higher stage tumors. Each bar shows the mean ± SEM (n = 49 for stage T1, n = 10 for stage T2, n = 2 for stage T3-T4, and n = 108 for noncancer controls). (d) ROC curves for APE1/Ref-1, the determination of different bladder tumor stages. (e) Urinary APE1/Ref-1 levels are higher in patients with muscle-invasive bladder cancer. Each bar shows the mean ± SEM (n = 157 for non-muscle-invasive bladder cancer [NMIBC], n = 12 for muscle-invasive bladder cancer [MIBC], and n = 108 for noncancer controls). (f) ROC curves for APE1/Ref-1, the determination of different of NMIBC and MIBC. (g) APE1/Ref-1 levels are higher in patients with previously recurrent tumors (n = 71 for previously recurrent tumors and n = 98 for nonrecurrent tumors). (h) ROC curves for APE1/Ref-1, the determination of recurrent bladder tumors. *Bars*: SEM. $^{***}p < 0.01$, significantly different from noncancer controls.

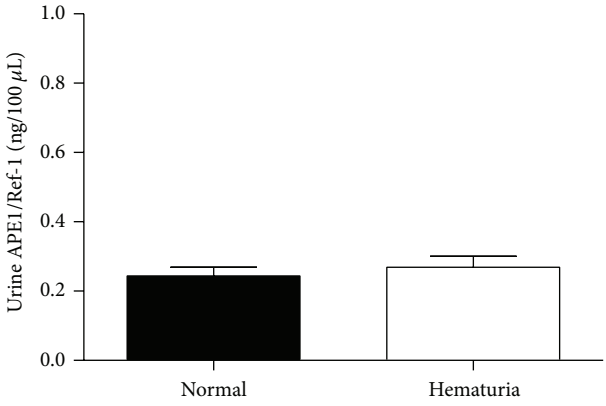

Figure 3: Urine APE1/Ref-1 levels in noncancer controls with hematuria (n = 30) did not differ significantly from the levels in noncancer controls without hematuria (n = 78). *Bars*: SEM. $p > 0.5$.

sample quantities, noninvasive sampling for repeated measurements, and less concern about pathogens transmission than with a blood sample. In general, the proteins in urine originate from glomerular filtration of blood, excretion from epithelial cells in the urinary tract, sloughing of epithelial cells and casts, and formation of urinary exosomes [22]. Changes in urine protein components and concentrations may directly indicate dysfunction of urothelial cells that line the uropoetic organs within the bladder. Therefore, measurement of urinary APE1/Ref-1 levels for BCa monitoring is a rational strategy. However, further studies are needed to determine the origin of urinary APE1/Ref-1 in the bloodstream or bladder itself, the mechanism of excretion into urine, and the meaning of the blood to urine APE1/Ref-1 ratio; urinary APE1/Ref-1 can be established as a new BCa biomarker. However, the results from our urinary APE1/Ref-1 analysis demonstrate an acceptable sensitivity, specificity, and reliability as a BCa diagnostic.

APE1/Ref-1 has been implicated in the development and progression of various cancers [10–12, 23]. Genetic variants of APE1/Ref-1 have been also studied to determine the relationship between specific polymorphisms and cancer susceptibility. Because of its frequency, the most well studied genetic variant is Asp148Glu; D148E [24]. Interestingly, APE1/Ref-1 has been shown to exhibit an atypical subcellular distribution pattern and cytoplasmic localization in many cancer types [25]. Based on our finding and those of previous reports, we propose that the high frequency and cytoplasmic localization of the genetic variant D148E APE1/Ref-1 in BCa cells are associated with the existence of this protein in urine.

In this study, we used frozen urine samples. To avoid alteration of the protein composition during frozen storage, we minimized the number of freeze-thaw cycles (1-2) by preparing small aliquot of each urine sample. Further, precipitates containing cellular debris, which may bias test results, were removed from the urine before storage. However, it is possible that freshly voided urine samples will yield different results. The cellular contribution of proteins derived from

bladder or renal tissues or squamous epithelial cells from the urethra or external genitalia also cannot be ruled out.

In our analysis, we carefully considered the effects of hematuria, urinary hemoglobin levels when APE1/Ref-1 levels in urine were monitored by ELISA, because many people with hematuria have been evaluated for BCa, and an APE1/Ref-1 ELISA needs to be performed with accuracy despite the presence of hemoglobin in urine. It was previously shown that the levels of urinary bladder tumor antigen (BTA) were highly correlated with urinary hemoglobin levels, indicating blood as a source of BTA in urine [26]. To exclude this possibility we confirmed the immune reaction of rh APE1/Ref-1 in hematuria samples, reconstituted by mixing urine and blood. The results, obtained using serially diluted rh APE1/Ref-1 in simulated hematuria samples, were almost completely implemented regardless of hemoglobin. Accurate quantitation of hemoglobin in hematuria may provide valuable insight into the clinical utility of APE1/Ref-1 as a BCa biomarker.

In conclusion, the overexpression of APE1/Ref-1 in BCa and its detection in blood [10] and urine solidify the potential for APE1/Ref-1 to serve as a BCa biomarker. The level of APE1/Ref-1 in urine as well as blood may be useful for identifying patients with BCa. Additionally, testing of urine APE1/Ref-1 is a convenient and noninvasive method that can distinguish BCa and thus reduce unnecessary and painful cystoscopy. Additional prospective cohort investigation using larger pools of samples, including samples from cases with various prognoses, is required to validate APE1/Ref-1 as a biomarker in the treatment of BCa.

Conflict of Interests

There is no potential conflict of interests to disclose.

Authors' Contribution

Sunga Choi and Ju Hyun Shin contributed equally to this paper.

Acknowledgments

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Review Article

Systemic Inflammatory Response Based on Neutrophil-to-Lymphocyte Ratio as a Prognostic Marker in Bladder Cancer

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A growing body of evidence suggests that systemic inflammatory response (SIR) in the tumor microenvironment is closely related to poor oncologic outcomes in cancer patients. Over the past decade, several SIR-related hematological factors have been extensively investigated in an effort to risk-stratify cancer patients to improve treatment selection and to predict posttreatment survival outcomes in various types of cancers. In particular, one readily available marker of SIR is neutrophil-to-lymphocyte ratio (NLR), which can easily be measured on the basis of absolute neutrophils and absolute lymphocytes in a differential white blood cell count performed in the clinical setting. Many investigators have vigorously assessed NLR as a potential prognostic biomarker predicting pathological and survival outcomes in patients with urothelial carcinoma (UC) of the bladder. In this paper, we aim to present the prognostic role of NLR in patients with UC of the bladder through a thorough review of the literature.

1. Introduction

Cancer is a leading cause of morbidity and mortality presenting multifactorial features affected by a variety of factors, including tumor-related and host (patient)-related factors. Until recently, predicting outcomes in cancer patients have mainly depended upon tumor characteristics, such as pathologic tumor stage and tumor grade. However, various host-related factors, including weight loss (cachexia), performance status, and systemic inflammatory response (SIR), have been suggested as potential prognostic indicators in cancer patients.

Since Virchow first described a possible connection between inflammation and cancer in 1876 after observing the presence of leukocytes within neoplastic tissues [1], clear evidence now supports the crucial role played by SIR in the development, progression, metastasis, and survival of malignant cells in most cancers [2]. Most solid malignancies trigger an intrinsic inflammatory response that builds up a protumorigenic microenvironment. Inflammation in the tumor microenvironment may promote angiogenesis, invasion, and metastasis via the signaling of tumor-promoting chemokines and cytokines (i.e., IL-1, IL-6, tumor necrosis factor- [TNF-] a, and IL-23), which are produced by innate immune cells (macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells) and adaptive ones (T and B lymphocytes) [1, 2]. Based on this background, in recent years many clinical studies have supported SIR as a meaningful predictor of survival outcomes in various types of cancers, including cancers of the lung [3–5], colorectum [6–11], gastrointestinal tract [12, 13], liver [14, 15], esophagus [16–18], breast [19–21], ovaries [22–24], cervix [25, 26], and pancreas [27]. In addition, the prognostic value of SIR has been vigorously assessed in urologic cancers, including prostate cancer [28–31], kidney cancer [32–34], and urothelial carcinoma (UC) (cancers of the bladder [35–48] and upper urinary tract (UUT) [49–60]).

UC is the second-most frequently diagnosed urologic malignancy. Clinical outcomes vary. A majority of UCs (90~95%) originate in the bladder, and UC of the UUT only accounts for 5~10% of all UCs. Radical cystectomy (RC) and radical nephroureterectomy (RNU), respectively, are applied

as the gold standard local treatment for muscle-invasive or high-risk, non-muscle-invasive UC of the bladder and UUT. However, in spite of these aggressive local approaches, long-term prognosis remains poor due to disease recurrence accompanied by local and/or distant metastasis [61–63]. These poor outcomes suggest a need for ongoing risk stratification and proper selection of multimodal treatment approaches, such as chemotherapy in the neoadjuvant or adjuvant setting. To address these issues, a number of studies have explored SIR-related biomarkers as potential predictors of oncologic outcomes in UC. Among these, NLR, defined as the ratio of absolute neutrophils to absolute lymphocytes, has recently gained considerable attention as a biomarker in urothelial carcinoma (UC) arising from the bladder or upper urinary tract (UUT).

In this paper, we reviewed the clinical studies dealing with SIR-related biomarkers in association with oncologic outcomes in UC, with a special focus on NLR.

2. SIR-Based Prognostic Scoring System

Potential hematological biomarkers representing SIR in cancer patients include C-reactive protein (CRP), albumin, Glasgow Prognostic Score (GPS), modified GPS (mGPS), and neutrophil-to-lymphocyte ratio (NLR). The association of these SIR-related biomarkers with oncological outcomes has been extensively studied by many investigators in many types of nonurologic cancers (Table 1). Because hematological tests are routinely performed in most cancer patients, these biomarkers may be used as easily measurable, objective, reproducible, robust, and inexpensive parameters able to express the severity of SIR in cancer patients.

CRP is a nonspecific but sensitive marker of the acute phase response and is expressed in selected tumor cells [64]. The biological basis for the correlation between expression of this marker, cancer risk, outcome, and survival is not completely understood. Several proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and TNF-a, expressed by the tumor environment induce CRP synthesis from the liver and other tissues [1, 2]. Based on many recent studies, it is now widely accepted that an elevated CRP value is a reliable indicator of poor prognosis in a variety of types of cancers [4, 8, 14, 16, 23, 28, 29, 31, 32, 65, 66].

Serum albumin, another marker of acute phase response to an inflammatory state, is generally used to assess nutritional status, severity of disease, disease progression, and prognosis [64]. Malnutrition and inflammation suppress albumin synthesis. In an adult, the normal range of serum albumin level is 3.5–5.0 g/dL. When levels drop below 3.5 g/dL, the condition is called hypoalbuminemia. The lower serum albumin concentration may be due to the production of cytokines such as IL-6, which modulate the production of albumin by hepatocytes [64]. Alternatively, TNF-a may increase the permeability of the microvasculature, thus allowing an increased transcapillary passage of albumin. Presence of micrometastatic tumor cells in the liver may induce the Kupffer cells to produce a variety of cytokines (IL-1, IL-6, and TNF-a), which may modulate albumin synthesis by hepatocytes [1, 2]. Thus, hypoalbuminemia is uncommon in early-stage cancer but as the disease progresses, albumin levels drop significantly and serve as good prognostic indicators in patients with various cancers [7, 19, 22, 67].

GPS and mGPS are inflammation-based prognostic scores developed by combining CRP and albumin to predict the clinical outcomes in cancer patients [68, 69]. GPS and mGPS, as routinely available, easily measured, and well standardized worldwide hematologic biomarkers, have subsequently been the subject of prognostic studies in wide variety of operable [13, 15, 18, 25, 27, 34] and inoperable [9, 10, 17, 20, 33] cancers. Indeed, these scoring systems have been extensively validated in various clinical scenarios and are now recognized to have prognostic value independent of tumor-based factors, such as pathologic tumor stage, tumor grade, lymphovascular invasion, and lymph node involvement.

It is also well recognized that SIR is related to changes in circulating white blood cells, especially an abnormal increase in neutrophils (neutrophilia) along with an abnormal decrease in lymphocytes (lymphocytopenia) [2, 64]. In light of this phenomenon under inflammatory conditions, NLR, being the ratio of neutrophils to lymphocytes, has gained considerable interest over the past decade not only as a potential prognostic factor associated with outcomes in a variety of cancers but also as a means of refining risk stratification of patients to treatment and predicting survival rates. Currently, NLR has been demonstrated to have significant prognostic value in urologic cancers, such as prostate [70] and renal cancer [71, 72], and also in cancers outside the urinary system [5, 11, 21, 24, 26].

3. SIR in Bladder Cancer

Prognosis in bladder cancer utilizes the same factors utilized for other types of cancers, including tumor-related factors, such as tumor stage, grade, lymphovascular invasion (LVI), and lymph node involvement (LNI) [61, 63]. However, all of these factors feature postoperative parameters. Given that SIR-related hematological biomarkers are easily obtained through pretreatment routine blood examination and have provided reliable prognostic information in other types of cancers, these biomarkers have been investigated in risk stratification for recurrence and mortality of patients with bladder cancer in both pre- and posttreatment settings. Several clinical studies have found an association between SIR-related hematological biomarkers, including CRP, albumin, and GPS, and oncologic outcomes of UC of the bladder (Table 3). In each different treatment setting, elevated CRP, defined as different cut-off (1.0 or 0.5 mg/dL),was significantly related to worse cancer-specific-survival (CSS) [35, 36]. One study demonstrated that in muscle-invasive bladder cancer (MIBC) patients with elevated CRP levels showed significantly more adverse pathologic features, such as extravesical disease (≥pT3), larger tumor size, lymph node involvement, and positive surgical margin prior to undergoing RC compared to patients with normal CRP levels. In addition, one-unit elevation in pre-RC CRP levels was significantly associated with a 20% increased risk of cancer-related death after RC [37]. In

Table 1: Clinical studies on the prognostic value of SIR-related hematological biomarkers in various types of cancers other than UC.

Study	Marker	Type of cancer	Threshold	Assessment period	Results
Parker et al. [22]	Albumin	Ovarian cancer	3.5 & 4.1 g/dL	Before operation	Low-albumin level (continuous value) was associated with worse OS
Lis et al. [19]	Albumin	Breast cancer	3.5 g/dL	Before operation	Low-albumin level (<3.5 g/dL) was related to higher death rate
Lai et al. [7]	Albumin	Colon cancer	3.5 g/dL	Before operation	Hypoalbuminemia (<3.5 g/dL) was associated with increased morbidity and mortality
Seebacher et al. [67]	Albumin	Endometrial cancer	4.21 g/dL or continuous	Before operation	Increased albumin level (continuous) was related to better DFS and PFS
Hashimoto et al. [14]	CRP	HCC	1.0 mg/dL	Before operation	Elevated CRP (>1) was significant predictor of worse OS and RFS
Lehrer et al. [28]	CRP	Prostate cancer	NA (continuous)	Before radiation	There was a significant correlation of CRP level with PSA
Crumley et al. [16]	CRP	Gastroesophageal cancer	1.0 mg/dL	Before operation	Elevated CRP (>1) was independent predictor of CSS
Jones et al. [4]	CRP	Lung cancer	0.4 mg/dL	Before operation	Elevated CRP (>0.4) was related to larger tumor size, advanced tumor stage, and incomplete resection
Karakiewicz et al. [32]	CRP	RCC	0.4 & 2.3 mg/dL	Before nephrectomy	Elevated CRP (>2.3) was an informative predictor of worse CSS
Beer et al. [29]	CRP	Metastatic prostate cancer	0.8 mg/dL	Before docetaxel based chemotherapy	Elevated CRP (>0.8) was a strong predictor of poor OS and lower PSA response to chemotherapy
Hefler et al. [23]	CRP	Ovarian cancer	1.0 mg/dL	Before surgery	Elevated CRP (>1.0 & continuous) was associated with postoperative residual tumor and worse OS
Shiu et al. [8]	CRP	Colorectal cancer	0.5 mg/dL	Before surgery	Elevated CRP (>0.5) was correlated with larger tumor size, higher stage, and poorer CSS
Crumley et al. [17]	GPS	Inoperable gastroesophageal cancer	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before nonsurgical treatment	High GPS was significant predictor of worse CSS
Al Murri et al. [20]	GPS	Metastatic breast cancer	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before non-surgical treatment	High GPS was significant predictor of worse CSS
Ramsey et al. [33]	GPS	Metastatic RCC	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before treatment	High GPS was significant predictor of worse CSS
Polterauer et al. [25]	GPS	Cervical cancer	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before surgery	High GPS was significant predictor of worse OS and DFS
Vashist et al. [18]	GPS	Esophageal cancer	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before surgery	High GPS was a strong prognosticator of perioperative morbidity and worse DFS and OS
Kinoshita et al. [15]	GPS	HCC	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before treatment	High GPS was independently associated with worse CSS
Leitch et al. [9]	mGPS	Colorectal cancer (operable or unresectable)	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before treatment	High mGPS was independently associated with worse CSS in patients with either operable or unresectable colorectal cancer
Jiang et al. [13]	mGPS	Gastric cancer	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before surgery	High mGPS was independently associated with worse OS irrespective of cancer stage

Table 1: Continued.

Study	Marker	Type of cancer	Threshold	Assessment period	Results
Ishizuka et al. [10]	mGPS	Unresectable colorectal cancer	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before chemotherapy	High mGPS (1/2) was an independent risk factor of poor CSS
La Torre et al. [27]	mGPS	Pancreatic cancer	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before surgery	High mGPS was independently associated with worse OS irrespective of cancer stage
Lamb et al. [34]	mGPS	RCC	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	Before surgery	High mGPS was significantly independent predictors of worse OS and CSS
Cho et al. [24]	NLR	Ovarian cancer	2.6	Before surgery	Positive NLR (>2.6) showed worse OS and DFS than negative NLR (<2.6)
Chua et al. [11]	NLR	Metastatic colorectal cancer	5	Before chemotherapy	Elevated NLR (>5) was independently associated with less clinical response to chemotherapy and worse OS and PFS
Azab et al. [21]	NLR	Breast cancer	Multiple cut-offs (1.8, 2, 45, 3, 33)	Before chemotherapy	High NLR (>3.3) was an independent significant predictor of all-cause mortality
Keizman et al. [70]	NLR	Metastatic CRPC	3	Before ketoconazole	Low NLR (≤3.0) was significantly associated with better PFS
Keizman et al. [71]	NLR	Metastatic RCC	3	Before sunitinib	Low NLR (≤3.0) was independent predictor of better response to sunitinib and favorable PFS and OS
Lee et al. [26]	NLR	Cervical cancer	1.9	Before treatment	High NLR (≥1.9) was related to more advanced stage and increased NLR (continuous) was an independent predictor of worse PFS and OS
Yao et al. [5]	NLR	Advanced lung cancer	2.63	Before chemotherapy	Low NLR (≤2.63) was independently associated with better clinical response to chemotherapy and favorable OS and PFS

RCC: renal cell carcinoma, HCC: hepatocellular carcinoma, CRP: C-reactive protein, GPS: Glasgow Prognostic Score, mGPS: modified Glasgow Prognostic Score, NLR: neutrophil-to-lymphocyte ratio, OS: overall survival, DFS: disease-free survival, PFS: progression-free survival, and CSS: cancer specific survival.

inoperable advanced bladder cancer, hypoalbuminemia and GPS 2 measured prior to chemotherapy were independently associated with shortened progression-free survival (PFS) and overall survival (OS), respectively [39]. Recently, Ku et al. developed a nomogram incorporating albumin, lymphocyte count, and platelet count to predict the probability of 5-year OS and disease-specific survival (DSS) after RC that demonstrated higher predictive accuracy than the existing staging system [46].

4. NLR in Non-Muscle-Invasive Bladder Cancer (NMIBC)

To date, few studies have assessed the association between NLR and the prognosis of NMIBC initially treated with transurethral resection of the bladder tumor (TURBT). Indeed, the evaluation of the prognostic role of NLR has been conducted with focus on MIBC patients undergoing RC or a mixed cohort of muscle-invasive and non-muscle-invasive tumors (Table 2). One recent study assessed the predictive value of preoperative NLR in 107 patients initially diagnosed with NMIBC following TURBT [47]. When applying each different cut-off point for NLR using the standardized cut-off finder algorithm, NLR > 2.41 and NLR > 2.43 were significantly associated with unfavorable disease progression and recurrence. Owing to the limited sample size of this study, further studies will be required to validate the role of NLR as a predictor for recurrence and progression in NMIBC.

5. NLR in Muscle-Invasive Bladder Cancer (MIBC)

In the past five years, the prognostic role of NLR in MIBC has been actively investigated in association with various oncological outcomes, including pathologic outcome, post-RC recurrence, and survival (Table 2). Several studies evaluated the association between NLR and post-RC survival outcomes [38, 40, 41, 44]. The cut-off point chosen to define an elevated NLR differed across studies, ranging from 2.5 to 3. Although one study reported no significant association between elevated NLR and OS [40], elevated NLR has been regarded as an independent predictor of RFS (recurrence-free survival), OS, and CSS in most studies [38, 41, 44]. One study reported that higher NLR values were observed in MIBC patients compared with NMIBC patients [42]. In addition, several studies demonstrated a significant correlation between a higher NLR and adverse pathologic outcomes, such as larger tumor size, pathological upstaging to locally advanced disease (pT3), and LNI after RC [41–44]. In locally advanced MIBC treated with neoadjuvant chemotherapy (NACH) prior to RC, continuous NLR decrease from before NACH to before RC was observed only in patients showing a pathological response after RC; therefore, sustained NLR decrease during NACH was suggested as a potential surrogate marker reflecting the effect of NACH [48]. The aforementioned studies mainly dealt with the prognostic value of NLR in the pretreatment setting. Interestingly, one recent study elucidated the influence of posttreatment NLR measured in the early post-RC period on oncologic outcomes [45]. The cut-off point of pre- and post-RC NLR (2.1 and 2.0, resp.) was differently determined according to each receiver operating characteristics (ROC) curve analysis. Similar to the aforementioned study results, elevated NLR after RC was also significantly associated with adverse pathologic outcomes, such as pT3/T4 disease, LVI, and LNI, and was an independent predictor of OS and CSS. Moreover, patients with perioperative continuous elevated NLR (2.1 ->2.0) showed worse OS and CSS compared with other change groups. Therefore, pre- and posttreatment NLR might have prognostic value in predicting postoperative survival outcome in patients with MIBC.

6. NLR in Upper Urinary Tract Urothelial Carcinoma (UTUC)

Similar to bladder cancer, the prognostic significance of other SIR-related hematological biomarkers, including CRP, albumin, and neutrophil count, has been proven to be reliable in terms of predicting adverse pathologic and survival outcomes following definitive surgery in UTUC [49, 51, 52, 55, 58]. In recent years, the prognostic role of NLR has also been vigorously assessed in UTUC [50, 53, 56, 57, 60] (Table 3). Although all of the studies involved cohorts of patients with operable UTUC, the threshold to determine elevated NLR levels was not uniform, ranging from 2.5 to 3. However, irrespective of the choice of NLR threshold, elevated NLR over the threshold was consistently correlated with adverse postoperative pathologic findings (high tumor grade, advanced tumor stage, LVI, and LNI) and worse survival outcomes following RNU.

7. Clinical Implications of SIR in Bladder Cancer

NMIBC can primarily be treated with TURBT. However, frequent recurrence (50~70%) and progression (10~20%) rates after TURBT are a major concern [61, 73]. Management of NMIBC might involve lifelong surveillance and place a considerable economic burden on patients. Currently, cystoscopy is the standard of care during the surveillance period. It is, however, invasive, and repeated cystoscopic examinations can cause substantial discomfort and pain to patients. Although investigators have developed various models to predict recurrence and progression after TURBT for NMIBC including nomogram, scoring systems, and risk tables [74–77], these models mainly incorporated tumor-related factors, such as tumors number, tumor diameter, T category, World Health Organization (WHO) tumor grade, and carcinoma in situ (CIS). Considering the significant correlation of elevated NLR with disease recurrence and progression in NMIBC [47], the addition of NLR to the existing prediction model may contribute to more accurate stratification of patients with NMIBC according to risk of recurrence and progression. Also, according to risk stratification based on pretreatment NLR values, selective cystoscopic examination and additional treatment, including intravesical Bacillus Calmette-Guérin (BCG) immunotherapy or chemotherapy, will be possible

Table 2: Clinical studies on the prognostic value of SIR-related hematological biomarkers in UC of the bladder.

Study	Marker	Publication year	Number of patients (NMIBC/MIBC)	Threshold	Assessment period	Main findings
Hilmy et al. [35]	CRP	2005	105 (76/29)	1.0 mg/dL	Before surgery (TURBT)	Elevated preoperative CRP (>1) was independently associated with worse CSS
Yoshida et al. [36]	CRP	2008	88 (0/88)	0.5 mg/dL	Before radiochemotherapy	Elevated preoperative CRP (≥0.5) was independent predictor of worse CSS
Gakis et al. [37]	CRP	2011	246 (0/246)	0.5 mg/dL or continuous	1–3 days before RC	Patients with elevated CRP (>0.5) showed advanced age, more extravesical disease, larger tumor size, node positive disease, and positive surgical margin and increased CRP (continuous) was independent predictor of worse CSS
Hwang et al. [39]	GPS, Albumin	2012	67 (0/67)	1.0 mg/dL (CRP) 3.5 g/dL (Albumin)	1 day before first chemotherapy cycle	Hypoalbuminemia (<3.5) and GPS 2 was independently associated with reduced PFS and OS, respectively
Ku et al. [46]	Albumin Neutrophil count Platelet count	2015	419 (173/246)	3.5 g/dL (Albumin) 7500/uL (Neutrophil) 400×10^5/ul (Platelet)	Before RC	Low albumin, high lymphocyte count, and high platelet count were significantly associated with worse OS and CSS
Gondo et al. [38]	NLR	2012	189 (62/127)	2.5	Before RC	Elevated NLR (≥2.5) was an independent predictor of worse DSS
Demirtaş et al. [40]	NLR	2013	201 (35/166)	2.5	Before RC	Elevated NLR (>2.5) was not associated with overall survival
Hermanns et al. [41]	NLR	2014	424	3	Before RC	Patients with elevated NLR (≥3) significantly showed more advanced pathologic tumor stage Elevated NLR (≥3) was significantly associated with RFS, OS, and CSS
Kaynar et al. [42]	NLR	2014	291 (192/99)	NA (continuous)	1 day before surgery (TURBT or RC)	Patients with MIBC showed significantly higher NLR value than those with NMIBC Also, higher NLR significantly correlated with advanced age, larger tumor size, and aggressive tumor invasiveness
Potretzke et al. [43]	NLR	2014	102 (31/71)	NA (continuous)	Before RC	NLR was significant predictor of pathological upstaging after RC; also, patients with pathological upstaging to ≥pT3 had a significantly greater NLR compared to patents who remained at ≤pT2
Viers et al. [44]	NLR	2014	899 (392/507)	2.7	Within 90 days before RC	Elevated NLR (≥2.7) was significantly associated with adverse pathologic finding (higher pathologic tumor stage, node positive, and larger tumor size); increased NLR (continuous) was independently associated with worse RFS, OS, and CSS

Table 2: Continued.

Study	Marker	Publication year	Number of patients (NMIBC/MIBC)	Threshold	Assessment period	Main findings
Mano et al. [47]	NLR	2015	107 (107/0)	2.41 (for progression) 2.43 (for recurrence)	Before TURBT	Elevated NLR (>2.41) showed more pT1 tumors and was significantly associated with disease progression; elevated NLR (>2.43) was independent predictor of disease recurrence
Seah et al. [48]	NLR	2015	26 (0/26)	NA	Before NACH, during NACH, and after RC	Significant NLR decrease from before NACH to before RC was observed in patients with pathological response after NACH and RC
Kang et al. [45]	NLR	2015	385	2.0 (postoperative) 2.1 (preoperative)	Within 1 month before RC and within 3 months after RC	Patients with elevated postoperative NLR (≥2.0) had higher rates of ≥pT3, LVI, and positive lymph node and elevated postoperative NLR (≥2.0) was an independent predictor of OS and CSS; also, patients with perioperative continuous elevated NLR (2.1–>2.0) showed worse OS and CSS compared with other change groups

CRP: C-reactive protein, GPS: Glasgow Prognostic Score, mGPS: modified Glasgow Prognostic Score, NLR: neutrophil-to-lymphocyte ratio, TURBT: transurethral resection of bladder tumor, RC: radical cystectomy, NACH: neoadjuvant chemotherapy, NMIBC: nonmuscle invasive bladder cancer, OS: overall survival, DSS: disease-specific survival, RFS: recurrence-free survival, and CSS: cancer specific survival.

Table 3: Clinical studies on the prognostic value of SIR-related hematological biomarkers in upper urinary tract urothelial carcinoma.

Study	Marker	Publication year	Number of patients	Threshold	Assessment period	Main findings
Saito et al. [49]	CRP	2007	130	0.5 mg/dL	Before surgery	Patients with elevated (>0.5) CRP showed higher hemoglobin, advanced tumor stage (≥pT3), positive lymph node, high grade, and LVI; moreover, elevated (>0.5) CRP was significant prognostic factor for DSS and RFS
Obata et al. [52]	CRP	2013	183	0.5 mg/dL	Before surgery	Patients with elevated (>0.5) CRP showed advanced tumor stage (≥pT3), LVI, and higher number of metastases; moreover, elevated (>0.5) CRP was significant prognostic factor for worse RFS and CSS
Tanaka et al. [58]	CRP	2014	564	Multiple cut-offs (0.5, 2.0 mg/dL)	Before surgery	Elevated CRP (0.5–2.0 or >2.0) level was an independent predictor of worse RFS and CSS relative to normal CRP (≤0.5); in elevated pre-CRP (>0.5) group, postoperative normalization of CRP (≤0.5) was an independent predictor of better CSS
Ku et al. [55]	Albumin	2014	181	3.5 g/dL	Before surgery	Hypoalbuminemia (<3.5) was a significant predictor of worse DSS and OS; also, scoring model incorporated albumin discriminated patients well according to risk of DSS and OS
Hashimoto et al. [51]	Neutrophil count	2013	84	4000/uL	Before surgery	Elevated neutrophil count (≥4000/uL) was an independent prognostic factor for worse RFS
Azuma et al. [50]	NLR	2013	137	2.5	Before surgery	Elevated (≥2.5) NLR was significantly associated with worse RFS and CSS; also, scoring model incorporated NLR discriminated patients well according to risk of RFS and CSS
Dalpiaz et al. [53]	NLR	2014	202	2.7	Before surgery	Elevated (≥2.7) NLR was significantly associated with worse OS and CSS
Luo et al. [56]	NLR	2014	234	3	Before surgery	Elevated (≥3) NLR was significantly associated with worse MFS and CSS; also, the use of a NLR of >3 further identified a poor prognostic group, especially in patients with pT3 for MFS and CSS
Tanaka et al. [57]	NLR	2014	665	3	Before surgery	Patients with elevated (>3) NLR significantly showed high tumor grade (Gr 3), advanced tumor stage, positive lymph node, and LVI; elevated (≥3) NLR was an independent risk factor for worse RFS and CSS; furthermore, addition of pre-NLR slightly improved the accuracies of the base model for predicting both RFS and CSS
Sung et al. [60]	NLR	2015	410	2.5	Before surgery	Elevated NLR (≥2.5) was independent predictor of worse PFS, OS, and CSS, along with elevated ESR

CRP: C-reactive protein, NLR: neutrophil-to-lymphocyte ratio, LVI: lymphovascular invasion, OS: overall survival, DSS: disease-specific survival, RFS: recurrence-free survival, MFS: metastasis-free survival, and CSS: cancer specific survival.

in patients with high-risk NMIBC, thereby reducing their economic burden and the potential discomfort caused by repeated cystoscopy.

In terms of MIBC, one significant challenge has been the limited, pretreatment, risk-stratification data that exists for patients undergoing RC. The well-established risk factors for recurrence and survival in MIBC included tumor-related factors, including pathologic tumor stage, pathologic tumor grade, CIS, LVI, and LNI [78–80]. Moreover, most predictive models (nomogram) predicting recurrence and survival in bladder cancer have been heavily based on postoperative pathologic factors, such as pathologic tumor stage, pathological grade, LVI, and LNI [81–83], with minimal consideration for associated host-related factors. Meanwhile, the accuracy of clinical staging in bladder cancer remains poor, reporting upstaging rate of 50% at RC specimen [84]. Thus, not enough data exists to facilitate appropriate patient counseling and guide clinical trial enrollment. As such, it is required to identify biomarkers that can assist with preoperative patient risk stratification and counseling. To achieve these goals, SIR-related hematological biomarkers can be a potential and promising factor. Assessment of SIR-related biomarkers in bladder cancer may be particularly relevant, because the inflammatory process seems to play an important role in the genesis and progression of, as well as mortality from, bladder cancer [1, 2]. Based on the previous study result [43], demonstrating a significant association between pretreatment elevated NLR and pathologic upstaging after RC, the performance of early cystectomy or NACH prior to RC might be considered in patients with pretreatment high NLR to attain tumor downstaging and improve postoperative survival. In addition, the pattern of change in NLR during NACH will be a valuable surrogate marker for monitoring and predicting pathological response to NACH [48]. Several studies reported the incorporation of SIR-related hematological biomarkers, such as CRP, NLR, albumin, and lymphocyte and platelet count, with a predictive model for survival outcomes in MIBC [37, 38, 46] or UTUC [50, 55, 57], improved predictive accuracy of the model, and consequently discriminated patients well according to risk stratification. It follows that pretreatment evaluation of NLR will be helpful in counseling patients about their prognosis.

A recent study revealed that the NLR value measured during the early postoperative period (from 1 to 3 months) after RC had a significant correlation with adverse oncological and survival outcomes [45]. Thus, postoperative NLR and the pattern of NLR change in the perioperative period may also provide valuable information in determining which patients should be referred for additional multimodal treatment, such as radiation and adjuvant chemotherapy.

The limitations of current NLR-associated studies in cancer are as follows. First, as mentioned earlier, there was no uniform cut-off point for NLR; each threshold was adopted according to a variety of statistical methodologies. Unlike tumor-related prognostic factors, including pathologic tumor stage and grade, NLR as a host-related factor can be affected by a variety of physiologic conditions, such as patients' comorbidities (hypertension and diabetes mellitus) and type of cancer, which can trigger immune response to cancer so that the establishment of definite NLR threshold may be difficult in consideration of these changeable physiologic conditions among cancer patients. Second, nearly all of the studies were both clinical and retrospective. Further large-scale prospective clinical or experimental animal (preclinical) research using a unified and robust statistical methodology will be required to determine the definite cut-off value of NLR and to discover the biological mechanisms supporting the correlation between NLR and oncologic outcomes in cancer patients.

8. Conclusion

Elevated NLR has shown a significant association with adverse oncologic and survival outcomes in patients with UC. Thus, NLR as a potential marker of SIR may become a promising tool in the management of patients with UC of the bladder and UUT, in terms of improved risk assessment for prognosis and guidance for treatment. Moreover, the ease and convenience of routine blood examinations in the clinical setting mean that NLR can be an objective, inexpensive, reproducible, and cost-effective measurement for the prediction of prognosis in UC. However, current NLR-related studies have not applied uniform NLR thresholds and thus require cautious interpretation because of many statistical methodological limitations. For the introduction of NLR into the clinical practice, rigorous attempts should be made in proper prospective study design.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Correlation and Significance of Urinary Soluble Fas and Vascular Endothelial Growth Factor in Bladder Urothelial Cancer

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Aim. To investigate the correlation and significance between the urine soluble Fas (sFas) and vascular endothelial growth factor (VEGF) expression in patients with urothelial bladder carcinoma (UC). *Methods*. The level of sFas was measured by enzyme-linked immunosorbent assay (ELISA) and the expression of VEGF protein in UC surgical specimens was screened by immunohistochemical method. These data were analyzed through SPSS 13.0 software. *Results*. The urinary sFas levels were significantly higher in the patients with UC than in those without cancer (168.0 ng/mL ± 84.6 versus 56.2 ng/mL ± 37.0; $P < 0.05$) and in the cases with a higher stage or grade than in those with a lower stage or grade (each $P < 0.05$). They had a positive relationship between the expression of VEGF protein and the pathological stage or grade in UC tissues (each $P < 0.05$). Spearman rank correlation test showed a significant correlation between sFas levels and VEGF expressions ($R = 0.882$, $P < 0.05$). *Conclusions*. The effects of sFas and VEGF may play important roles together in the occurrence and progression of UC. Joint detection of urine sFas plus VEGF protein may provide valuable solutions to improve the diagnosis and treatment of UC.

1. Introduction

Urothelial bladder carcinoma (UC) is the most common malignancy of the urinary tract in China. The cancer has the highest recurrence rate of any solid tumor. Unfortunately, the recurrence and progression of the disease may not be well predicted in individual patient using the clinical and pathological parameters including tumor size, grade, stage, the multiplicity of lesions, and prior recurrence rate [1]. Therefore, it is critical to identify new biochemical factors. Obviously, determining the potential tumor markers of UC can indicate the different risks of recurrence and progression in patients; it even provides a targeted diagnosis and treatment for the patients. And tumor markers subsequently help to design the individualized therapeutic strategy, which will greatly improve the survival time and quality of life of patient with UC. At present, however, none of tumor markers for urothelial carcinoma have been applied to clinical practice alone and proved by multicenter and large scale study.

Previous studies have shown that apoptosis plays a very important role in the occurrence and development of bladder cancer [2], which provides a new way for diagnosis and treatment of UC. For apoptosis, the Fas/Fas ligand (FasL) system is a key regulator. Fas is a transmembrane cell surface receptor that triggers cell death upon binding to FasL, which is an anchored cell membrane protein exposed to the extracellular space [3]. Fas can be found in two forms, the transmembrane form and the soluble form. Soluble Fas (sFas) can bind to membrane-bound FasL, thus blocking the binding of the ligand to the Fas receptor, preventing apoptosis induction in the target cell, and enhancing the immunosuppressive effects of tumors. Some research shows that sFas can be detected in

the urine or serum of the bladder cancer patients. We [4, 5] and Svatek et al. [6] noted that the urinary soluble Fas levels in patients undergoing surveillance for urothelial carcinoma were higher in cancer patients than the control group and that the urinary sFas was an independent predictor of bladder cancer recurrence and invasiveness in patients who had a past history of UC.

Interestingly, vascular endothelial growth factor (VEGF) can inhibit apoptosis [7]. VEGF, which is considered the most important of the angiogenic stimulators during tumor angiogenesis, has been implicated as a major survival (anti-apoptotic) factor [8]. Angiogenesis, the growth of new blood vessels from the existing ones, is a marker of aggressiveness, which is known to play a leading role in the survival, proliferation, and metastatic potential of malignant tumors [8, 9]. It is a normal physiological process in fetal development and wound healing. But, in cancer, it is essential for tumor growth and metastatic spread. VEGF is expressed in bladder tumors, and the increased expression of VEGF is associated with higher tumor stage and progression [8–10]. However, to the best of our knowledge, no studies have applied combined detection of urinary soluble Fas and VEGF in UC.

In this study, we investigated the correlation and significance between the urine soluble Fas and vascular endothelial growth factor expression in patients with UC.

2. Methods

2.1. Patients. In this study, patients suffering from UC were recruited at random between January 2008 and May 2013 at the First Hospital of Shijiazhuang (Shijiazhuang, China) and the Affiliated Hospital to Chengde Medical College (Chengde, China). The classification of tumor stage and grade was made according to both 2002 TMN classification and World Health Organization (WHO) criteria (2004 version). None of the patients underwent preoperative adjuvant chemotherapy or radiotherapy. Cancer-free controls, without a history or family history of cancer or other genetic diseases, frequency matched by gender and age (±3 years), were recruited from individuals who visited the same hospitals. Subjects who suffered from renal insufficiency, heart failure, upper urinary tract tumors, intraoperative vesical perforation, or significant hematuria were excluded. We analyzed 82 patients (51 males and 31 females) with UC and their mean age was 58.8 years (range: 21–84). Of the 82 urothelial bladder carcinoma cases, with size from 0.2 to 4 cm in diameter, 56 were histologically diagnosed as nonmuscle invasive bladder cancers (NMIBC), 26 were muscle invasive bladder cancers (MIBC), 42 were low grade papillary urothelial cancers (LGPUC), and 46 were high grade papillary urothelial cancers (HGPUC). The control cohort consisted of 82 cancer-free individuals including 15 with benign prostatic hyperplasia, 17 with stones, 20 with nonspecific urinary tract infections, and 30 healthy volunteers.

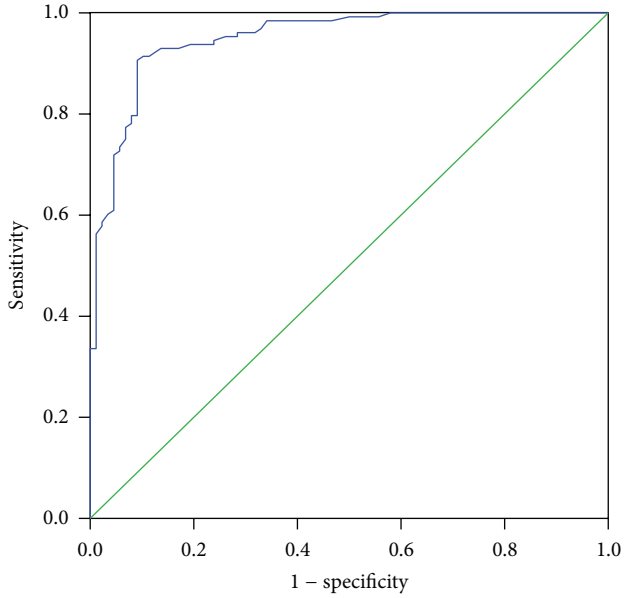
The study was approved by the Ethical Review Committee of First Hospital of Shijiazhuang and conformed to ethical guidelines of 1964 Declaration of Helsinki. All UC patients and cancer-free controls provided written informed consent before enrollment.

2.2. Enzyme-Linked Immunosorbent Assay. The first morning voided urine sample (10–20 mL) was obtained for measurement of sFas from preoperative patients and controls. Urine samples were centrifuged immediately at 2,000 g for 10 min and then frozen at −70°C until assayed. Levels of sFas in urine samples were quantified by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., USA) according to the manufacturer's protocol and expressed as ng/mL. Every sample was measured 3 times and the mean was calculated for data analysis.

2.3. Immunohistochemical Study and Scoring. The immunohistochemical (IHC) study was carried out on formalin-fixed, paraffin-embedded tissues. Serial sections of the tumor tissues were obtained from archived paraffin-embedded tissue blocks. In all cases, the primary pathological diagnosis was confirmed by hematoxylin and eosin (HE) staining; then slides were stained for VEGF. In brief, all slides were deparaffinized in xylene and then rehydrated in ethanol. Subsequently, all sections were treated with 3% hydrogen peroxide for 5 min and heated in a citrate buffer using a steam cooker and then were incubated with the polyclonal primary antibody (VEGF, Boster Biological Technology, Ltd., China; 1 : 100 dilution) at 37°C for 2 hours. Secondary antibody and streptavidin-biotin complex (Boster Biological Technology, Ltd., China) were then applied at 37°C for 20 min, respectively. After rinsing, the slides were stained with diaminobenzidine as chromogen and counterstained with routine hematoxylin.

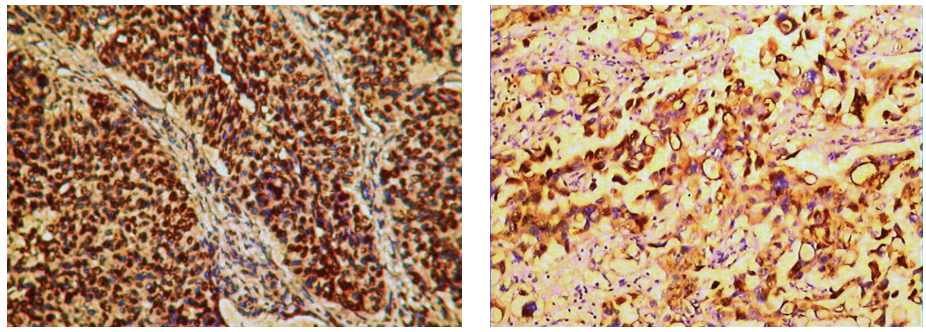
We used a semiquantitative analysis system to determine the VEGF immunostaining score according to the percentage and intensity of positive cells among less than 500 cells [9]. For percentage, 0~4 scores represent <5%, 5~25%, 26~50%, 51~75%, and >75% of the labeled cells, respectively. For the intensity, 0~3 scores indicate none, weak, middle, and strong staining, respectively. Multiplication of both scores decided the final score (VEGF score), ranging within 0~12. A double-blind analysis was performed by two independent pathologists. Each sample was scored twice. If the final scores had a more than 3-point discrepancy, a second evaluation would be performed. Using − ~+++, VEGF score = 0, 4 > VEGF score > 0, 8 > VEGF score ≥ 4, and VEGF score ≥ 8, respectively.

2.4. Statistical Analysis. Clinical and pathological data were gathered from each patient. Patient data were analyzed, and descriptive statistics were used to summarize the study population characteristics. Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., USA). The data of urinary soluble Fas level were presented as median ± interquartile range (M ± IQR). Differences in continuous variables were tested by Mann-Whitney U test. In order to derive the most appropriate sFas cutoff value for use in diagnosing UC, a receiver operating characteristic (ROC) curve was constructed. The value that maximized the difference between sensitivity and the false positive rate (Youden index = sensitivity + specificity − 1) was selected as the cutoff value.



Positive if greater than or equal to	Sensitivity	1 − specificity
44.5000	1.000	0.727
85.0000	0.914	0.114
87.5000	0.914	0.102
91.0000	**0.906**	**0.091**
96.0000	0.898	0.091
97.5000	0.875	0.091
743.0000	0.000	0.000

Figure 1: ROC curve of urine levels of sFas in the diagnosis of UC patients.



(a) (b)

Figure 2: Representative examples of VEGF in UC. Positive VEGF immunoreactivity is detected in the membrane or cytoplasm of cancer cells. Original magnifications: ×40 (a) and ×100 (b).

The correlations of variables were evaluated with Spearman rank correlation test. All statistical analyses were two-sided with P value of less than 0.05 defined as statistically significant difference.

3. Results

The ELISA technique was employed to determine urinary sFas concentration; the intra-assay coefficient of variation was 6.09%. The Mann-Whitney U test results showed that sFas levels were significantly higher in the urine of UC patients than in those without cancer (168.0 ng/mL ± 84.6 versus 56.2 ng/mL ± 37.0; $U = 333.5$, $P = 0.000$). On the basis of ROC curve, the area under the curve (AUC) was 0.928 (95% CI 0.887–0.969; Figure 1). By ELISA, the sensitivity and specificity of sFas for the patients of UC were 85.3% and 88.2%, respectively ($\chi^2 = 73.593$; $P = 0.000$), if the cutoff value of sFas was selected as 91.0 ng/mL according to Youden index (Figure 1).

The association between the clinicopathological characteristics and urinary sFas levels and VEGF expressions is shown in Table 1. By Mann-Whitney U test, the urinary sFas level was significantly higher in cases with a higher stage or grade than in those with a lower stage or grade (each $P < 0.05$), while it did not differ depending on gender or age (each $P > 0.05$).

Positive expression of VEGF was membrane staining or strong cytoplasmic reactivity (Figure 2). In 82 cases, the number of positive expressions of VEGF was 61 (74.4%). By the method of Spearman rank correlation test, they had a positive relationship between the expression of VEGF protein and the pathological stage or grade (each $P < 0.05$), so the positive rate of VEGF increased gradually with the progression of the pathological stage or histological grade of bladder cancer (Table 2). Compared with the VEGF negative expression group, the level of sFas was elevated in the VEGF positive expression group ($P = 0.000$, Table 1). And then the Spearman rank correlation test showed a significant

Table 1: Clinicopathological characteristics and urinary sFas levels and VEGF expressions of the UC patients.

Variables	n	Urinary sFas (ng/mL) M ± IQR	P value
Gender			0.312
Male	51	177.3 ± 94.2	
Female	31	158.0 ± 68.8	
Age (years)			0.741
≤60	62	168.1 ± 72.0	
>60	20	162.5 ± 86.0	
Grade			0.039
LGPUC	42	157.0 ± 78.3	
HGPUC	40	179.6 ± 91.2	
T stage			0.000
NMIBC	56	139.0 ± 71.3	
MIBC	26	230.5 ± 113.2	
VEGF expression			0.000
Negative	21	92.5 ± 41.3	
Positive	61	194.0 ± 99.5	

NMIBC: nonmuscle invasive bladder cancer; MIBC: muscle invasive bladder cancer; LGPUC: low grade papillary urothelial cancer; HGPUC: high grade papillary urothelial cancer.

Table 2: The correlation of VEGF expression and the pathological stage and grade in the UC patients.

	VEGF expression (n)				R value	P value
	−	+	++	+++		
Grade					0.735	0.001
LGPUC	14	4	14	10		
HGPUC	7	8	9	16		
T stage					0.823	0.001
NMIBC	19	6	20	11		
MIBC	2	6	3	15		

NMIBC: nonmuscle invasive bladder cancer; MIBC: muscle invasive bladder cancer; LGPUC: low grade papillary urothelial cancer; HGPUC: high grade papillary urothelial cancer.

correlation between the urinary sFas levels and the VEGF expression ($R = 0.882$, $P = 0.000$, Table 3).

Table 3: Urinary sFas value in different VEGF groups of the UC patients.

VEGF expression	n	Urinary sFas (ng/mL) M ± IQR	R value	P value
−	21	95.0 ± 36.5	0.882	0.000
+	12	129.5 ± 49.5		
++	23	158.5 ± 82.8		
+++	26	380.5 ± 327.5		

4. Discussion

In recent years, the research based on Fas/FasL system has become one of the important advances in the field of molecular biology [11, 12], and higher sFas levels in the serum have been investigated in various cancer types [4, 13, 14]. Mizutani et al. [15] reported elevated sFas levels in the serum of patients with bladder cancer and suggested an association of elevated sFas level with poor prognosis in these patients. Conversely, Perabo et al. [16] argued that serum sFas seemed useless as a tumor marker and that it had limited relevance in laboratory investigations of bladder cancer. There are few data currently available in the literature concerning urinary sFas in bladder cancer patients [5–7, 17]. In the present study, we found that urinary sFas levels in UC patients were higher in cancer patients than the control group. Compared with current test, the detection of urine soluble Fas by ELISA has its advantages because of a higher sensitivity and specificity and lower cost. Then, according to our results, sFas continued to rise with the increase of the aggressiveness of bladder cancer, and it may be used as an indicator of the malignant degree of bladder cancer. We speculate that high-level expression of sFas protein in UC may be involved in the pathogenesis and progression of the disease, leading to the initiation of cell apoptosis and causing bladder cancer cells insensitivity to postoperative intravesical instillation and therefore more likely causing tumor recurrence. However, the exact mechanism remains unclear.

There are four main forms of VEGF, each one with a variety of functions, including recruitment and mitogenic stimulation of endothelial cells [18]. Level of VEGF has been shown to influence recurrence and survival in urothelial carcinoma [9, 10]. According to the results of this study, VEGF expression can be detected in both invasive and noninvasive disease, and the increased expression of VEGF was associated with increasing tumor stage or grade of bladder cancer, which means VEGF plays an important role in the progression of bladder cancer and its expression may serve as an important prognostic indicator.

Furthermore, we found for the first time that the levels of sFas were elevated with the increase of the expression of VEGF, so the effects of sFas inhibiting the apoptosis and VEGF activating angiogenesis have a close relationship with the occurrence and progression of UC. Soluble Fas and VEGF may play important roles together in the occurrence and progression of urothelial bladder cancer and interact with each other via signal transduction network and then influence biological behavior of the tumor, but the specific mechanism needs further research. This study also suggests that joint detection of urine sFas plus VEGF protein has a practical value for evaluating progression and prognosis of UC, which will provide valuable solutions to improve the diagnosis and treatment of bladder cancer.

In terms of limitations, since this study was a preliminary investigation, the number of patients was relatively small. We reckon the statement that soluble Fas level is higher in the high grade patients which was premature unless cases with comparable mass and different grade were compared. In the meantime, the origin of sFas in urine is unknown even though this has no visible effect on the final evaluation.

In conclusion, the results showed that urinary sFas levels in UC patients were higher in cancer patients than the control group. In the meantime, VEGF expression can be detected

in both invasive and noninvasive disease, and the increased expression of VEGF is associated with increasing tumor stage or grade of bladder cancer. Furthermore, we found that the level of sFas was elevated with the increase of the expression of VEGF, so the effects of sFas and VEGF may play important roles together in the occurrence and progression of urothelial bladder cancer. This study also suggests that joint detection of urine sFas plus VEGF protein will provide valuable solutions to improve the diagnosis and treatment of bladder cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Steroid Hormone Receptor Signals as Prognosticators for Urothelial Tumor

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There is a substantial amount of preclinical or clinical evidence suggesting that steroid hormone receptor-mediated signals play a critical role in urothelial tumorigenesis and tumor progression. These receptors include androgen receptor, estrogen receptors, glucocorticoid receptor, progesterone receptor, vitamin D receptor, retinoid receptors, peroxisome proliferator-activated receptors, and others including orphan receptors. In particular, studies using urothelial cancer tissue specimens have demonstrated that elevated or reduced expression of these receptors as well as alterations of their upstream or downstream pathways correlates with patient outcomes. This review summarizes and discusses available data suggesting that steroid hormone receptors and related signals serve as biomarkers for urothelial carcinoma and are able to predict tumor recurrence or progression.

1. Introduction

Bladder cancer, which is mostly urothelial carcinoma, is one of the most frequently diagnosed neoplasms, with estimated 429,800 new cases and 165,100 deaths which occurred in 2012 worldwide [1]. Patients with superficial urothelial tumor suffer from its recurrence with occasional progression to muscle invasion after transurethral surgery. In contrast, those with muscle-invasive tumor often develop disease progression or metastatic tumor despite more aggressive treatment. Cystoscopy which is an invasive and relatively expensive procedure is the "gold standard" for the detection of bladder cancer [2–4]. Urine cytology is a highly specific, noninvasive adjuvant test widely utilized with cystoscopy for both screening/initial diagnosis of bladder cancer and surveillance of tumor recurrence [5]. There are also several urine-based markers/tests, such as nuclear matrix protein 22 (NMP22), bladder tumor antigen (BTA), and UroVysion, which are useful for detecting urothelial tumors and may thus be substitutes of cystoscopy and/or cytology [6–9]. However, none of these markers or tests have demonstrated a significant association with prospective tumor recurrence or disease progression in patients with urothelial cancer.

Epidemiological and clinical studies have indicated that men have a significantly higher risk of bladder cancer, whereas women tend to have more aggressive tumors [1, 10–15]. These observations have prompted investigations of steroid hormones and their receptor signals, especially androgens/estrogens and androgen/estrogen receptors (AR/ER), in bladder cancer, which have demonstrated their critical roles in tumorigenesis and tumor progression [16–18]. Accordingly, bladder cancer is now considered as an endocrine-related neoplasm. Additionally, studies have identified a variety of molecules or pathways regulated by steroid hormones and their receptor signals in bladder cancer cells. These findings have also provided novel therapeutic targets for urothelial carcinoma.

Recent evidence has thus indicated the involvement of nuclear receptor-mediated signals in urothelial cancer outgrowth. These receptors include AR, ERα, ERβ, glucocorticoid receptor (GR), progesterone receptor (PR), vitamin D receptor (VDR), retinoid receptors (e.g., retinoic acid receptor (RAR) and retinoid X receptor (RXR)), and peroxisome proliferator-activated receptors (e.g., PPARγ) as well as orphan receptors. More importantly, recent studies have assessed the prognostic significance of steroid hormone

receptor signals and related pathways in urothelial tumors. In this paper, we mainly review immunohistochemical studies showing associations between alterations of steroid hormone receptors in urothelial tumors and patient outcomes. Furthermore, we highlight several molecules regulated by AR and/or ER signals in bladder cancer cells, which may contribute to the development of diagnostic and/or prognostic biomarkers.

2. Androgens and AR

Using cell line and animal models, androgens have been shown to promote urothelial carcinogenesis and cancer progression via the AR pathway [16, 17, 19–26]. Specifically, androgen deprivation inhibited tumor development in male rodents treated with a bladder carcinogen *N*-butyl-*N*-4-hydroxybutyl nitrosamine (BBN) [21, 23]. Furthermore, BBN completely failed to induce bladder cancer in AR knockout mice [23]. AR signals have also been found to downregulate the expression of P450 CYP4B1 [27], UDP-glucuronosyltransferases (UGTs) [28], and GATA3 [29], all of which are known to prevent urothelial tumorigenesis. Meanwhile, androgen deprivation resulted in inhibition of bladder cancer cell proliferation and invasion [23–26, 30–33]. Recent clinical studies have also suggested that androgen deprivation therapy for prostate cancer prevents bladder cancer development [34] and recurrence [35].

Immunohistochemical studies have demonstrated that the positive rates of AR expression in bladder or upper urinary tract (UUT) urothelial tumors range from 13% to 55%, which is significantly lower than that in nonneoplastic urothelial tissues [25, 30, 36–46] (Table 1). However, two studies showed no AR expression in normal urothelium [30, 43]. Similarly, most of the studies showed downregulation of AR expression in high-grade and muscle-invasive tumors, compared with low-grade and non-muscle-invasive tumors, respectively [25, 30, 36, 39–41, 45].

Prognostic significance of AR expression in urothelial tumors remains controversial. Despite the promoting effects of AR signals on tumorigenesis, two studies showed a significant correlation [44] and a tendency [30], respectively, between AR expression and lower risks of bladder tumor recurrence. In contrast, AR expression correlated with the progression of bladder tumors [40, 43], while others did not reveal its prognostic significance in patients with bladder cancer. Additionally, none of the immunohistochemical analyses in UUT tumors have demonstrated strong correlations of AR expression with their outcomes.

3. Estrogens and ERs

Both stimulatory and inhibitory effects of estrogens on urothelial cancer outgrowth, which appear to be cell-specific and dependent on the functional activity of ERα and ERβ, have been documented [16, 18–20, 47–50]. For instance, significantly higher incidence of bladder cancer was observed in BBN-treated ERα knockout female mice, compared with wild-type female littermates, suggesting the preventive role of ERα in bladder cancer development [51]. Selective ER modulators, such as tamoxifen and raloxifen, were also shown to inhibit the growth of bladder cancer cell lines expressing ERβ [47, 49]. Nonetheless, estrogens promoted the cell proliferation of a urothelial cancer line predominantly via the ERα pathway as well as that of primary urothelium line predominantly via the ERβ pathway [50].

Immunohistochemistry has detected ERα protein only in a small subset (e.g., 1–5%) of bladder cancer specimens [43, 52–54] (Table 1). Of note, in a study using a quantitative polymerase chain reaction (PCR) method, *ERα* gene was found to be positive in all the 10 tumors examined, which was even stronger (2.77-fold) than in matched normal tissues [50]. Our immunohistochemical analyses showed higher positive rates in bladder (27% [40]) and UUT (18% [46]) tumors, compared with those in other studies described above. In contrast to the findings in PCR analysis [50], elevated levels of ERα protein expression were detected in nonneoplastic urothelium, compared with urothelial cancer [40, 46, 53]. At least two of the immunohistochemical studies also demonstrated that ERα expression was downregulated in higher grade or stage tumors [40, 53]. However, no studies have identified the prognostic values of ERα in patients with urothelial tumor.

ERβ protein expression was reported to be positive in 22–76% of urothelial tumors, which was significantly lower than the positive rates in nonneoplastic urothelial tissues, in some of the studies [30, 40, 41, 46, 52, 55, 56] (Table 1). More recently, Tan et al. [54] demonstrated that all the 410 bladder tumors examined were immunoreactive for ERβ. There was significant upregulation [40, 44, 52, 55] or downregulation [56] of ERβ expression seen in higher grade or more invasive tumors. Elevated ERβ expression in bladder cancers was also found to correlate with higher risks of tumor recurrence and/or progression [30, 40, 57], and ERβ positivity was an independent predictor of tumor progression [30]. Conversely, a strong association between ERβ overexpression and favorable prognosis was demonstrated [44, 54].

4. Glucocorticoids and GR

The relationship between glucocorticoids and urothelial tumorigenesis is debatable. A population-based case-control study showed that prolonged oral glucocorticoid use was at an increased risk of developing bladder cancer [58], presumably due to immunosuppression. In contrast, our preclinical studies have revealed that glucocorticoids directly mediate GR activity in bladder cancer cells and that GR functions as a tumor suppressor [59, 60]. Natural or synthetic glucocorticoids, such as corticosterone, prednisone, and dexamethasone, strongly inhibited bladder cancer cell invasion and metastasis via inactivating nuclear factor- (NF-) κB. However, treatment with dexamethasone resulted in an increase in bladder cancer cell viability and a decrease in apoptosis particularly that was induced by a cytotoxic agent, cisplatin, suggesting induction of chemoresistance by glucocorticoids. It is thus likely that GR signals, apart from glucocorticoid-induced immunosuppression, have dual roles in bladder cancer: suppression of tumor progression versus induction of cell proliferation. It should also be mentioned that the action of glucocorticoids is often complex and is generally dependent on a balance of transactivation and transrepression of GR

Table 1: Immunohistochemical studies for the expression of steroid hormone receptors in urothelial carcinoma specimens.

Author, year [reference]	Receptor	N	Location	Nontumor (nonneoplastic urothelium) versus tumor			Tumor grade			Tumor stage			Prognostic significance (P value)
				Non-Tumor	Tumor	P value	LG	HG	P value	NMI	MI	P value	
Boorjian et al., 2004 [36]	AR	49	Bladder	86%	53%	0.001*	89%	49%	0.055*	75%	21%	0.002*	NA
Boorjian et al., 2009 [25]	AR	55	Bladder	NA	44%	0.06	NA	NA	NA	59%	33%	0.095*	NA
Kauffman et al., 2011 [37]	AR	59	Bladder	84%	Roughly half	<0.001	NA	NA	NA	NA	NA	0.028 (NMI > MI)	NS
Mir et al., 2011 [38]	AR	472	Bladder	NA	13%	NA	12%	13%	0.83	9%	15%	0.058	NS
Rau et al., 2011 [39]	AR	93	UUT	NA	NA	NA	NA	NA	0.074 (LG > HG)	NA	NA	0.001 (stage II > I)	0.568
Tuygun et al., 2011 [30]	AR	139	Bladder	0% (M)	51%	<0.001*	64%	37%	0.002*	60%	21%	<0.001*	0.095 (RFS) 0.110 (PFS)
Miyamoto et al., 2012 [40]	AR	188	Bladder	80%	42%	<0.001	55%	36%	0.023	51%	33%	0.018	0.071 (PFS/MI)
Shyr et al., 2013 [41]	AR	83	UUT	NA	55%	NA	69%	47%	0.070*	63%	44%	0.114*	NA
Jing et al., 2014 [42]	AR	58	Bladder	NA	53%	NA	55%	50%	0.724	49%	69%	0.195	NA
Mashhadi et al., 2014 [43]	AR	120	Bladder	0%	22%	<0.001	NA	NA	<0.001	NA	NA	<0.001	0.02
Nam et al., 2014 [44]	AR	169	Bladder	NA	37%	NA	39%	33%	0.269	43% (Ta) 30% (T1)	NA	0.048 (Ta vs T1)	0.001 (RFS) 0.288 (PFS)
Williams et al., 2015 [45]	AR	297	Bladder	NA	25%	NA	NA	NA	NA	33% (Ta/Tis)	19% (T1–3)	0.010*	NA
Williams et al., 2015 [45]	AR	43	UUT	NA	16%	NA	NA	NA	NA	NA	NA	NA	NA
Kashiwagi et al., 2015 [46], and unpublished data	AR	99	UUT	58%	20%	<0.001	33%	18%	0.177	14%	24%	0.301	NS
Shen et al., 2006 [52]	ERα	224	Bladder	NA	1%	NA	NA	NA	NA	NA	NA	NA	NA
Bolenz et al., 2009 [53]	ERα	198	Bladder	NA	5%	0.06	NA	NA	NA	NA	NA	0.004 (OC > non-OC)	NS
Miyamoto et al., 2012 [40]	ERα	188	Bladder	50%	27%	<0.001	38%	23%	0.048	35%	19%	0.014	NS
Mashhadi et al., 2014 [43]	ERα	120	Bladder	2%	3%	0.67	NA	NA	NA	NA	NA	NA	NA
Tan et al., 2015 [54]	ERα	410	Bladder	NA	4%	NA	NA	NS	NA	NA	NS	NA	NA
Kashiwagi et al., 2015 [46], and unpublished data	ERα	99	UUT	40%	18%	0.001	27%	17%	0.465	11%	23%	0.183	NS

Table 1: Continued.

Author, year [reference]	Receptor	N	Location	Nontumor (nonneoplastic urothelium) versus tumor			Tumor grade			Tumor stage			Prognostic significance (P value)
				Non-Tumor	Tumor	P value	LG	HG	P value	NMI	MI	P value	
Croft et al., 2005 [55]	ERβ	92	Bladder	NA	11–22%*	NA	6–12%* (G1-2)	17–33%* (G3)	0.021–0.177*,**	5–9%* (Ta)	16–33%* (≥T1)	0.010–0.098*,**	NA
Shen et al., 2006 [52]	ERβ	224	Bladder	NA	63%	NA	58% (G1-2)	70% (G3)	0.085	54%	80%	<0.001	NA
Kontos et al., 2010 [56]	ERβ	111	Bladder	93%	76%	0.041*	95% (G1-2)	56% (G3)	<0.001*	83% (T1)	54%	0.011*	NA
Tuygun et al., 2011 [30]	ERβ	139	Bladder	0% (M) 36% (F)	27–30%**	<0.001*	22–26%**	31–34%**	0.44–0.59**	24–26%**	36–42%**	0.16–0.24**	0.114 (RFS) 0.025 (PFS)
Miyamoto et al., 2012 [40]	ERβ	188	Bladder	89%	49%	<0.001	29%	58%	<0.001	34%	67%	<0.001	0.007 (PFS/LG) ≤0.001 (PFS) 0.007 (CSS/MI)
Kauffman et al., 2013 [57]	ERβ	72	Bladder	NA	NA	<0.001 (N < T)	NA	NA	NA	NA	NA	NS	0.030 (RFS) 0.0018 (CSS)
Shyr et al., 2013 [41]	ERβ	83	UUT	NA	43%	NA	44%	43%	1.000*	51%	47%	0.815*	NA
Nam et al., 2014 [44]	ERβ	169	Bladder	NA	31%	NA	27%	41%	0.043	22% (Ta) 42% (T1)	NA	0.004 (Ta vs T1)	0.004 (RFS) 0.014 (PFS)
Tan et al., 2015 [54]	ERβ	410	Bladder	NA	100%	NA	100%	100%	NS	100%	100%	NS	0.055/0.087 (CSS)
Kashiwagi et al., 2015 [46], and unpublished data	ERβ	99	UUT	85%	63%	0.001	73%	61%	0.402	65%	63%	1.000	NS
Ishiguro et al., 2014 [62]	GR	149	Bladder	96%	87%	0.026	96%	81%	0.011	96%	74%	<0.001	0.025 (RFS/NMI) 0.030 (PFS/MI) 0.067 (CSS/MI)
Kashiwagi et al., 2015 [46], and unpublished data	GR	99	UUT	84%	63%	0.001	53%	64%	0.563	62%	63%	1.000	NS
Bolenz et al., 2009 [53]	PR	198	Bladder	NA	0%	NA	NA	NA	NA	NA	NA	NA	NA
Mashhadi et al., 2014 [43]	PR	120	Bladder	2%	4%	0.48	NA	NA	NA	NA	NA	NA	NA
Tan et al., 2015 [54]	PR	410	Bladder	NA	0%	NA	0%	0%	NS	0%	0%	NS	NA
Kashiwagi et al., 2015 [46], and unpublished data	PR	99	UUT	13%	16%	0.487	7%	18%	0.453	14%	18%	0.779	0.041 (CSS/pT3-4)
Hermann and Andersen, 1997 [70]	VDR	26	Bladder	NA	100%	NA	100%	100%	0.043* (LG < HG)	100%	100%	0.051* (NMI < MI)	NA
Sahin et al., 2005 [71]	VDR	105	Bladder	67%	86%	0.02	81% (G1)	91% (G2-3)	NS	85% (Ta) 87% (T1)	NA	NS	0.001 (PFS)
Inamoto et al., 2010 [85]	Nurr1	145	Bladder	0% (high)	100% 65% (high)	NA	35% (high; G1-2)	92% (high; G3)	<0.001	47% (high)	92% (high; including T1b)	<0.001	<0.001 (RFS)*** <0.001 (CSS)***

AR: androgen receptor; ER: estrogen receptor; GR: glucocorticoid receptor; PR: progesterone receptor; VDR: vitamin D receptor; UUT: upper urinary tract; NA: not analyzed; M: males; F: females; LG: low-grade; HG: high-grade; NMI: non-muscle-invasive; MI: muscle-invasive; OC: organ-confined; RFS: recurrence-free survival; PFS: progression-free survival; CSS: cancer-specific survival; NS: not significant

*We calculated the two-tailed P values using Fisher's exact test.

**Two criteria.

***Cytoplasmic expression.

that involve therapeutic effects of glucocorticoids and adverse effects associated with glucocorticoid therapy, respectively. Recently, we found that compound A, a plant derivative known to function as a GR agonist as well as an AR antagonist, induced only GR transrepression in bladder cancer cells and more efficiently inhibited tumor growth than dexamethasone or an antiandrogen flutamide [61].

Our immunohistochemical studies in bladder [62] and UUT [46] tumors showed that most of nonneoplastic urothelial tissues expressed the GR, which was downregulated in urothelial neoplasms (Table 1). GR expression was also significantly reduced in high-grade or muscle-invasive bladder tumors, compared with low-grade or non-muscle-invasive tumors [62]. However, this was not seen in UUT tumors [46]. In addition, loss of GR expression was found to correlate with recurrence of non-muscle-invasive bladder tumors and progression of muscle-invasive bladder tumors in univariate analyses [62]. Multivariate analysis identified low GR expression as a predictor for recurrence of non-muscle-invasive bladder tumors (hazard ratio (HR) = 2.252; $P = 0.034$) and progression of muscle-invasive bladder tumors (HR = 3.690; $P = 0.077$). However, the levels of GR expression were not significantly associated with the prognosis of the patients with UUT tumor in our study [46].

5. Progestogens and PR

A case-control study demonstrated significant decreases in bladder cancer incidence in multiparous women or women with oral contraceptive use [63]. In a study using a transgenic model for bladder cancer, multiparous female mice developed significantly smaller tumors than nulliparous females [64]. These observations imply benefits of not only estrogens but also progestogens for preventing the development of urothelial tumors.

Hormone-binding assay showed that 1 of 3 noninvasive and 3 of 3 advanced urothelial tumors were positive for PR [65]. An immunohistochemical study also demonstrated PR expression in the urothelium in 18 of 20 bladders from male children aged 1–12 [66]. Nonetheless, two subsequent immunohistochemical studies in 198 [53] and 410 [54] bladder cancer specimens failed to detect PR signals (Table 1). In another study of bladder tumors, the positive rates of PR were 2% and 4% in nonneoplastic urothelium and carcinoma tissues, respectively [43]. We recently showed that 13% of nonneoplastic urothelial tissues from the UUT and 16% of UUT tumors were immunoreactive for PR [46]. There was no significant difference in PR expression between low-grade versus high-grade or non-muscle-invasive versus muscle-invasive UUT tumors. Interestingly, in our study, PR positivity in pT3 or pT4 UUT tumors was strongly associated with disease-specific mortality.

6. Vitamin D and VDR

Low serum levels of vitamin D have been implicated in the risk of bladder cancer [67]. *VDR* gene polymorphism resulting in reduction of receptor activity has also been correlated with higher incidence of bladder cancer [68]. Furthermore, vitamin D was shown to prevent bladder tumorigenesis in rats treated with a carcinogen N-methylnitrosourea as well as to inhibit cell growth of VDR-positive bladder cancer lines [69]. Thus, VDR signals appear to play a protective role in bladder tumor outgrowth.

VDR was found positive immunohistochemically in 86–100% of bladder tumors [70, 71] (Table 1). In contrast to the above findings, however, upregulation of VDR expression was seen in high-grade and muscle-invasive tumors, compared with low-grade and non-muscle-invasive tumors, respectively, in one of the studies [70]. Strong VDR expression was significantly associated with lower progression-free survival and cancer-specific survival rates.

7. Retinoic Acids and Retinoid Receptors

The preventive effects of retinoic acids, including vitamin A and its derivatives, on bladder cancer development have been assessed. A recent meta-analysis involving 25 studies demonstrated a significant inverse association between dietary intake of vitamin A/retinol and bladder cancer risk [72]. Preclinical studies also showed that retinoids inhibited bladder carcinogenesis in animals treated with BBN [73] and cell proliferation of bladder cancer lines [74].

In a study using a PCR-based method, all of the nonneoplastic bladders were found to express the retinoid receptors [75]. However, some of muscle-invasive bladder cancers lost RARα (60%), RARγ (20%), and RXRα (40%), while they were positive in all non-muscle-invasive tumors. RARβ2 was positive in 50% of non-muscle-invasive tumors and 40% of muscle-invasive tumors. In addition, methylated RARβ was frequently found in bladder cancer tissues and urine samples from bladder cancer patients [76–78], suggesting its utility as a urine marker. Specifically, the sensitivity of RARβ for tumor detection was higher than that of urine cytology (68% versus 46% for all cases; 67% versus 11% for grade 1 tumors) [77].

8. PPARs

There has been a link between the use of pioglitazone, a PPAR agonist prescribed as a hypoglycemic drug, and bladder cancer risk [79]. Indeed, treatment with a PPARγ agonist rosiglitazone or PPARγ overexpression resulted in significant increases in bladder cancer cell migration and invasion [80]. Earlier studies conversely showed that PPARγ agonists inhibited bladder cancer cell growth [81, 82]. Of note, there appear to be multiple mechanisms for inducing antitumor effects of PPARγ agonists, some of which are independent of PPARγ signals [83]. Additionally, *in situ* hybridization showed that *PPARγ* gene was often amplified in bladder cancer specimens [80, 81].

9. Orphan Nuclear Receptors

Okegawa et al. recently demonstrated up- or downregulation of a variety of orphan nuclear receptor genes in bladder cancer tissues, compared with paired normal bladders [84]. Of these receptors, hepatocyte nuclear factor 4γ (HNF4G) was most frequently elevated in tumors and its overexpression

Table 2: Molecules regulated by sex hormone receptor signaling in urothelial carcinoma.

	Associated receptor signaling	Effect on urothelial carcinogenesis and/or cancer progression	Hormone effect	Reference
CD24	AR	Stimulation	Upregulation	[88]
β-catenin	AR	Stimulation	Upregulation	[92, 93]
Slug	AR	Stimulation	Upregulation	[42]
EGFR	AR	Stimulation	Upregulation	[31]
ERBB2	AR	Stimulation	Upregulation	[31]
Akt	AR/ERα	Stimulation	Upregulation/downregulation	[31, 51]
ERK1/2	AR	Stimulation	Upregulation	[31]
Cyclin D1	AR	Stimulation	Upregulation	[26]
Cyclin D3	AR	Stimulation	Upregulation	[29]
Cyclin E	AR	Stimulation	Upregulation	[29]
FGFR3	AR	Stimulation	Upregulation	[29]
UGT1A	AR/ERβ	Inhibition	Downregulation/upregulation (SVHUC)	[28, 110]
p53	AR	Inhibition	Downregulation	[29, 118]
p21	AR	Inhibition	Downregulation	[29, 118]
PTEN	AR	Inhibition	Downregulation	[29]
c-myc	AR	Stimulation	Upregulation	[29]
Bcl-xL	AR	Stimulation	Upregulation	[26]
MMP-9	AR	Stimulation	Upregulation	[26]
ELK1	AR	Stimulation	Up-regulation	[33]
GATA3	AR/ERβ	Inhibition	Downregulation/upregulation (SVHUC)	[29]
INPP4B	ERα	Inhibition	Upregulation	[51]

AR: androgen receptor; ER: estrogen receptor.

promoted tumor growth in both *in vitro* and *in vivo* [84]. Nurr1 was also often overexpressed in bladder cancers [84, 85], which correlated with the promotion of bladder cancer cell migration [85]. Immunohistochemistry of Nurr1 in bladder cancer specimens showed significant increases in its expression levels in higher grade/stage tumors [85] (Table 1). Moreover, high cytoplasmic Nurr1 expression, but not total expression, was an independent prognosticator of cancer-specific mortality (HR = 4.894; $P < 0.001$) [85]. Similarly, Nur77 was overexpressed especially in muscle-invasive bladder cancers [84, 86]. However, Nur77 activation correlated with retardation of bladder tumor growth in cell line and animal models [86, 87].

10. Molecules Regulated by Steroid Hormone Receptor Signaling

Increasing evidence suggests the involvement of upstream pathways as well as downstream targets of steroid hormone receptor-mediated signals in the development and progression of urothelial cancer. Table 2 summarizes such molecules directly or indirectly regulated by AR and/or ER signals. The following are key molecules that androgens/estrogens have been shown to up- or downregulate via the AR/ER pathways in bladder cancer cells.

10.1. CD24. AR signals activate CD24, a glycoprotein and a cell adhesion molecule, in bladder cancer cells [88]. In animal models, CD24 overexpression and knockdown resulted in stimulation and inhibition, respectively, of the development of primary bladder cancer and its metastasis [88, 89]. Immunohistochemical analyses in bladder cancer specimens [89–91] have revealed that CD24 is expressed exclusively in tumor cells, but not in surrounding stromal cells. These studies also showed higher levels of CD24 expression in grade 2-3 tumors (74%) than in grade 1 tumors (28%; $P < 0.001$) [90], in ≥pT3 tumors than in ≤pT2 tumors ($P = 0.036$) [91], or in metastatic tumors (93%) than in primary tumors (75%; $P = 0.006$) [89]. Furthermore, elevated CD24 expression was associated with recurrence of non-muscle-invasive tumors ($P < 0.001$ for all cases or grades 2-3; $P = 0.042$ for grade 1) [90] or cancer-specific mortality in patients with muscle-invasive tumor ($P < 0.001$) [91] in univariate settings. However, CD24 was not an independent prognosticator for muscle-invasive bladder cancers (HR = 1.12; $P = 0.84$) [91].

10.2. β-Catenin. AR signals activate Wnt/β-catenin signaling in bladder cancer cells [92, 93]. β-Catenin, as a key component of the Wnt signaling pathway, is a multifunctional protein and is known to activate target genes, such as the protooncogene *c-myc*, the cell cycle activator *cyclin D1*, and the *epidermal growth factor receptor* (*EGFR*). Using an animal model for bladder cancer, β-catenin was shown to induce tumorigenesis, and androgen-mediated AR signals appeared to synergize with β-catenin [93]. There are conflicting data as to the correlation of β-catenin staining in bladder cancer

specimens with tumor aggressiveness. Consistent with the findings in other studies [94, 95], we observed downregulation of membranous β-catenin expression in bladder cancer, compared with nonneoplastic urothelium [92]. In addition, loss or reduced expression of membranous β-catenin, as well as nuclear accumulation of β-catenin as a hallmark of Wnt/β-catenin activation, correlated with higher tumor grade, more advanced tumor stage, and/or worse patient outcomes [42, 92, 94, 96]. Coexpression of nuclear β-catenin and AR in bladder cancer cells was also noted [42, 92].

10.3. Slug. Androgens were shown to upregulate Slug expression in bladder cancer cells, which could subsequently induce epithelial-to-mesenchymal transition through the activation of Wnt/β-catenin signaling [42]. Slug expression was significantly upregulated in high-stage bladder cancers (e.g., non-muscle-invasive 27% versus muscle-invasive 77%, $P = 0.023$ [42]; lymph node-negative 58% versus lymph node-positive 89%, $P = 0.012$ [97]; non-muscle-invasive 23% versus muscle-invasive 77%, $P = 0.04$ [98]), whereas there were no statistically significant differences in Slug expression between low-grade and high-grade tumors in these 3 studies. Prognostic significance of Slug expression in bladder tumors was not seen or was not assessed in these studies.

10.4. EGFR/ERBB2. Activation of the EGFR family, such as EGFR and ERBB2, is known to involve bladder tumorigenesis and cancer progression. Accordingly, the efficacy of targeted therapy directed at EGFR signals has been assessed in bladder cancer [99–104]. We demonstrated that androgen upregulated the expression of EGFR and ERBB2 as well as the levels of phosphorylation of their downstream proteins AKT and extracellular signal-regulated kinase- (ERK-) 1/2 via the AR pathway in bladder cancer cells [31]. EGF could also induce bladder cancer cell proliferation via modulating AR signals [32]. Alterations of the EGFR family, such as protein overexpression and gene amplification or mutation, have been extensively studied in bladder cancer specimens, providing mixed results regarding their prognostic values [104–109]. For instance, some studies suggested that ERBB2 overexpression was a poor prognostic factor, while others did not. Nonetheless, ERBB2 was found to be overexpressed in muscle-invasive bladder cancers in most of the studies.

10.5. UGT1A. UGT1A, a major phase II drug metabolism enzyme, plays a critical role in detoxifying bladder carcinogens. In a normal urothelial cell line SVHUC as well as in normal mouse bladders, androgens/estrogens decreased/increased the expression levels of UGT1A and its subtypes via the AR/ERβ pathways, respectively [28, 110]. An initial immunohistochemical study showed that 6 of 19 bladder tumors lost UGT1A, while benign tissues consistently expressed it [111]. Our immunohistochemical staining subsequently showed reduced expression of UGT1A in 145 urothelial neoplasms, compared with paired nonneoplastic urothelial tissues, as well as inverse correlations between UGT1A levels and tumor grade or pT stage [110]. Decreased UGT1A expression was also strongly associated with the progression of high-grade non-muscle-invasive tumors ($P = 0.038$) or worse cancer-specific survival in patients with muscle-invasive tumor ($P = 0.016$), and the latter was an independent prognosticator (HR = 3.413; $P = 0.010$) [110]. In addition, the expression of UGT1A was positively and negatively correlated with the levels of ERα and ERβ, respectively.

10.6. ELK1. ELK1, a member of the ETS-domain family of transcription factors, is known to involve cell proliferation, cell cycle control, and apoptosis via regulating the expression of a variety of genes, including *c-fos* protooncogene. We recently demonstrated that androgens activated ELK1 in bladder cancer cells and promoted the proliferation of only ELK1-positive cells and the migration/invasion of both ELK1-positive and ELK1-negative cells [33]. Androgens also failed to significantly induce AR transcriptional activity in ELK1 knockdown bladder cancer cells. Our immunohistochemical staining showed significant increases in the expression of ELK1 and phospho-ELK1 (an activated form of ELK1) in bladder tumors, compared with nonneoplastic urothelial tissues [33]. The expression of ELK1/phospho-ELK1 versus AR was significantly correlated. While there were no significant correlations between the levels of ELK1 or phospho-ELK1 and tumor grades or stages, phospho-ELK1 positivity precisely predicted the recurrence of non-muscle-invasive tumors in a univariate setting ($P = 0.043$) as well as a worse outcome of muscle-invasive tumors in both univariate ($P = 0.045$ for disease progression; $P = 0.008$ for cancer-specific mortality) and multivariate (HR = 2.693; $P = 0.021$ for cancer-specific mortality) settings. Subsequent immunohistochemistry in bladder cancer specimens from patients who received neoadjuvant chemotherapy revealed that phospho-ELK1 positivity strongly correlated with chemoresistance [112]. Indeed, ELK1 inactivation resulted in enhancement of the cytotoxic activity of cisplatin in bladder cancer cells [112].

10.7. GATA3. GATA3, a member of the GATA family of zinc-finger transcription factors, has recently been recognized as a urothelial marker and its immunohistochemistry has therefore been widely used in diagnostic surgical pathology [113–115]. Using SVHUC cells with carcinogen challenge, we demonstrated that GATA3 strongly prevented neoplastic transformation of urothelial cells [29]. GATA3 knockdown in SVHUC exposed to the chemical carcinogen resulted in downregulation of the molecules that play a protective role in bladder tumorigenesis, such as UGT1A, PTEN, p53, and p21, and upregulation of oncogenic genes, such as c-myc, cyclins D1/D3/E, and FGFR3. Additionally, similar to the findings in UGT1A described above, androgens/estrogens down/upregulated GATA3 expression in nonneoplastic urothelial cells via the AR/ERβ pathways, respectively [29]. GATA3 knockdown in bladder cancer lines also resulted in promotion of cell invasion and migration as well as induction of the expression of their related molecules, such as MMP-2 and MMP-9 [116], while androgens did not significantly change the levels of GATA3 expression in these cells [29]. Our immunohistochemical data showed that GATA3 was positive in 98% of nonneoplastic urothelial tissues versus 86% of urothelial neoplasms as well as in 98% of low-grade and/or non-muscle-invasive tumors versus 72–80% of high-grade

and/or muscle-invasive tumors [117]. In tumors, there were strong correlations between GATA3 expression versus AR overexpression, ERα overexpression, or loss of ERβ expression. We also demonstrated that patients with GATA3-positive muscle-invasive tumor had a significantly higher risk of disease progression in a univariate setting ($P = 0.048$) and, in this subgroup, strong GATA3 expression was correlated with tumor progression (HR = 2.435; $P = 0.052$) or cancer-specific survival (HR = 3.673; $P = 0.040$) in a multivariate setting [117].

10.8. Inositol Polyphosphate 4-Phosphatase Type II (INPP4B). INPP4B has been recognized as a tumor suppressor of several types of malignancies, such as breast and prostate cancers, but its role in bladder cancer remained unclear. In bladder cancer cells, estrogens were shown to upregulate INPP4B via the ERα pathway, resulting in inhibition of AKT activity and cell growth [51]. Chromatin immunoprecipitation assay further revealed that ERα could bind to a putative estrogen response element region of the INPP4B promoter in bladder cancer cells. Immunohistochemistry showed that INPP4B was positive in 62% of bladder tumors, which was significantly lower than in benign urothelial tissues (87%; $P < 0.001$) [51]. Similarly, 75% of low-grade versus 53% of high-grade tumors ($P = 0.016$) as well as 74% of non-muscle-invasive versus 44% of muscle-invasive tumors ($P < 0.001$) were INPP4B-positive. There was also a positive correlation between INPP4B expression and ERα expression. However, no prognostic significance of INPP4B expression in bladder tumors has been demonstrated.

11. Conclusion

Mounting evidence suggests that steroid hormone receptor-mediated signals play a critical role in urothelial tumorigenesis and cancer progression. Various molecules, as downstream targets, have also been shown to be modulated by these signals. Immunohistochemical studies in surgical specimens have identified significant differences in the expression levels of several steroid hormone receptors and their related proteins between nonneoplastic urothelium versus urothelial tumor and between low-grade/non-muscle-invasive versus high-grade/muscle-invasive urothelial tumors. More importantly, although the underlying mechanisms of how steroid hormone receptors and related signals regulate urothelial tumor outgrowth remain far from being fully understood, the available data support that these can serve as biomarkers of urothelial tumors, especially their prognosticators. Further investigation of steroid hormone receptors as well as other molecules directly or indirectly regulated by steroid hormones may help develop not only better strategies for the management of urothelial tumors but also more reliable biomarkers.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Predictive Markers for the Recurrence of Nonmuscle Invasive Bladder Cancer Treated with Intravesical Therapy

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High recurrence rate is one representative characteristic of bladder cancer. Intravesical therapy after transurethral resection is often performed in patients with nonmuscle invasive bladder cancer (NMIBC) to prevent recurrence. Bacillus Calmette-Guérin (BCG) and several anticancer/antibiotic agents, such as mitomycin C and epirubicin, are commonly used for this therapy. BCG treatment demonstrates strong anticancer effects. However, it is also characterized by a high frequency of adverse events. On the other hand, although intravesical therapies using other anticancer and antibiotic agents are relatively safe, their anticancer effects are lower than those obtained using BCG. Thus, the appropriate selection of agents for intravesical therapy is important to improve treatment outcomes and maintain the quality of life of patients with NMIBC. In this review, we discuss the predictive value of various histological and molecular markers for recurrence after intravesical therapy in patients with NMIBC.

1. Introduction

Urothelial cancer of the bladder is a common disease throughout the world. Approximately two-thirds of all bladder cancers (BCs) are considered nonmuscle invasive bladder cancer (NMIBC) at diagnosis [1]. Transurethral resection (TUR) of the bladder is the standard therapy to remove cancerous tissue from patients with NMIBC. Unfortunately, BC frequently recurs after TUR. In fact, up to 70% of patients with NMIBC experience local BC recurrence after receiving the appropriate treatment. The risk of BC recurrence and progression increases in high grade BC [2]. Intravesical therapy is recommended to reduce the risk of recurrence and progression in these patients. In recent years, various therapeutic agents have been examined in preclinical and clinical trials for use in post-TUR adjuvant intravesical therapy [3, 4]. The most commonly used therapeutic agents include Bacillus Calmette-Guérin (BCG) and a variety of chemotherapeutic agents, even now.

Intravesical immunotherapy with BCG is the most effective therapy to reduce the recurrence and progression of NMIBC and most guidelines recommend BCG therapy for patients with NMIBC [5]. However, intravesical BCG therapy can cause adverse effects, ranging from lower abdominal discomfort and cystitis to bladder atrophy and sepsis. In addition, nearly 40% of patients do not respond to intravesical BCG therapy [6]. A number of patients with NMIBC reject BCG therapy because of the high failure rates and severe adverse effects associated with the therapy. Intravesical therapy with chemotherapeutic or antibiotic agents is another popular therapeutic option. Adverse effects associated with these treatment options are rare and mild. However, some patients in the intermediate- or high-risk groups could be at higher risks of recurrence and progression than if they received intravesical BCG therapy. Therefore, accurate predictions of anticancer effects associated with each intravesical therapy are important when deciding treatment strategies in patients with NMIBC.

In this review, we discuss the prognostic value of various types of predictive factors for recurrence in patients with NMIBC treated with adjuvant intravesical therapy after TUR. Many reports have been published and well-written reviews exist on this topic. However, many of them involve analyses of intravesical BCG therapy. In addition, most of these reviews were published nearly fifteen years ago. Certainly, there are only few new and dramatic topics in this field.

However, intravesical therapy with chemotherapeutic agents or BCG continues to be the most commonly used and effective therapy for patients with NMIBC. Therefore, we made special efforts to summarize these treatments based on reports from the past decade. There are many reports on predictive markers in urine samples [7–9]. However, in this review, we discuss the results obtained from tissue samples because intravesical therapy is usually performed after TUR.

The most popular and effective agent for post-TUR intravesical therapy is BCG. Although various types of chemotherapeutic or antibiotic agents are used for this therapy, most studies investigated the anticancer effects of mitomycin C (MMC), epirubicin, and cytarabine (Ara-C). MMC is a chemotherapeutic agent that acts by inhibiting DNA synthesis. Epirubicin is an anthracycline antibiotic agent that demonstrates minimal transurothelial absorption [10]. Ara-C is an antagonist of pyrimidine metabolism that has low incidences of local and/or general adverse effects [11]. Gemcitabine, paclitaxel, and some other newly developed agents have also been investigated in preclinical and clinical trials [3, 4, 12]. We pay special attention to BCG, MMC, and epirubicin in this review because these are currently the most popular agents and most reports regarding predictive markers for cancer recurrence in adjuvant intravesical therapy used these agents.

Numerous studies investigated how to optimize intravesical therapy in patients with NMIBC by examining different durations, timings, and regimens [13]. However, no single best method has been determined. Intravesical therapies vary widely in dosage, duration, and the presence or absence of maintenance therapy even if the same agent was used. Thus, biases and differences in the evaluation of cancer-related and immune-related molecules in human tissues are inevitable. In addition, there is a report that BCG efficacy in Japanese population tended to show decreasing nonrecurrence rates with time whereas nonrecurrence rate in western countries increased each year [14]. Furthermore, in the Asian population, recurrence-free survival between intravesical BCG and intravesical MMC therapy was not significantly different [15]. Therefore, this review is limited by the lack of uniformity in therapeutic policies, evaluation methods, and races analyzed. However, we believe that the information in this review will still be useful for discussing treatment and observation strategies in patients with NMIBC.

2. Clinical Background and Pathological Features

Most investigators and urologists initially studied whether certain characteristics of patients (e.g., age, gender, body mass index, and information on adverse effects) could predict clinical outcomes and recurrence after adjuvant intravesical therapy. Most of these studies suggest that these characteristics cannot predict anticancer effects and outcomes after intravesical BCG therapy [21, 30, 31]. Various pathological features and endoscopic tumor characteristics were also analyzed for potential prognostic capabilities in recurrence after intravesical therapy. However, these factors also failed to predict the response and time to recurrence [31]. The expression levels of cancer-related molecules, immune responses, and gene polymorphisms were also analyzed in patients as the next potential prognostic resources. Before discussing the prognosis of BCG therapy in patients with NMIBC, we should note the difference between BCG refractoriness and BCF relapse. Recurrence-free survival and overall survival of BCG-refractory patients are significantly worse compared to those of BCG-relapsing patients [32]. Therefore, further detailed studies with rigid distinction may be essential in the future.

3. Cell Proliferation

Cell proliferation is an important regulator of tumor growth, progression, and outcome in patients with NMIBC [33, 34]. Therefore, many studies investigated the relationship between recurrence and cell proliferation indices measured using Ki-67-positive cells (Ki-67 labeling index; Ki-67 LI) [16, 35, 36]. However, a consensus on the value of Ki-67 for predicting recurrence has yet to be reached. For example, patients with a high Ki-67 LI have significantly worse recurrence-free survival after intravesical BCG therapy than those with a low Ki-67 LI [16, 36]. However, while a significant predictive value was detected in a univariate analysis, it was not detected in a multivariate analysis. Another report demonstrated that the Ki-67 LI did not predict recurrence in intravesical BCG therapy [19]. On the other hand, Chen et al. [18] reported that a high Ki-67 LI (>25%) was a significant predictor of recurrence after intravesical therapy with MMC and epirubicin ($P = 0.001$) in 72 patients with pTa or pT1. Ki-67 LI was also identified as an independent prognostic indicator in a multivariate analysis (risk ratio, 2.021; 95% confidence interval, 1.018–4.010; $P = 0.044$). When a similar analysis was performed, high Ki-67 LI (≥20%) was a significant predictive factor for progression-free survival in both univariate and multivariate analyses ($P = 0.006$ and $P = 0.042$, resp.) [17]. However, in this study, Ki-67 LI did not significantly correlate with recurrence even in a univariate analysis [17]. No conclusion can be reached about the prognostic roles of Ki-67 LI for adjuvant MMC and epirubicin therapies because these two studies used different protocols for intravesical therapy, chose different cut-off values for the Ki-67 LI, and examined different pathological features. Thus, Ki-67 LI cannot be considered a useful predictor of recurrence after intravesical therapy. A summary of the predictive values of Ki-67 LI for recurrence after intravesical therapy is shown in Table 1. On the other hand, there was a report regarding proliferating cell nuclear antigen (PCNA) and recurrence in patients with NMIBC treated with intravesical therapy [37]. This study showed that rates for PCNA-positive cancer cells were 52.6% (10/19) in recurrent and 78.9% (15/19) in nonrecurrent cases. Although a significant difference of PCNA was not detected in recurrence, the number of patients was relatively small ($n = 19$).

4. Apoptosis and Apoptosis-Related Molecules

The induction of cell death by apoptosis is an important anticancer effect of intravesical therapy [38]. Therefore,

Table 1: Predictive value for recurrence in cancer-related factors and molecules.

Variable	Patients		Agent	*P* value for recurrence		Year/reference
	N	Background		Univariate	Multivariate	
Ki-67	92	Tis, T1G3	BCG	0.015		2009/[16]
	129	T1G3	MMC	0.517		2010/[17]
	72	Ta, T1	MMC/epirubicin	0.011	0.044	2012/[18]
	61	T1G3	BCG	0.677		2013/[19]
P53	53	Tis, Ta, T1	BCG	0.741		2007/[20]
	27	T1G3	BCG	0.92		2009/[21]
	129	T1G3	MMC	0.452		2010/[17]
	61	T1G3	BCG	0.794		2013/[19]
	134*	T1 HG	BCG	0.830		2015/[22]
pRb	53	Tis, Ta, T1	BCG	0.580		2007/[20]
	27	T1G3	BCG	0.037		2009/[21]
	61	T1G3	BCG	0.951		2013/[19]
Survivin	74**	Tis, Ta, T1	BCG/MMC	0.003	0.02	2007/[23]
	78	Ta and T1	BCG	0.009		2012/[24]
	78	Ta and T1	BCG	0.043		2012/[24]
p27	61	T1G3	BCG	0.822		2013/[19]
p63	134*	T1 HG	BCG	0.129		2015/[22]
ΔNp63	134*	T1 HG	BCG	<0.001	* * *	2015/[22]
PTEN	61	T1G3	BCG	0.306		2013/[19]
FGFR3	61	T1G3	BCG	0.355		2013/[19]
Ezrin	92	Tis, T1G3	BCG	0.041		2009/[16]
CD9	61	T1G3	BCG	0.760		2013/[19]
MVD	26	Ta, T1	BCG	<0.0001	0.0011	2011/[25]
AT1R	26	Ta, T1	BCG	<0.0001	0.0012	2011/[25]
VEGF	72	Ta, T1	MMC/epirubicin	0.010	0.036	2015/[26]

BCG: Bacillus Calmette-Guérin; MMC: mitomycin C; Rb: retinoblastoma; PTEN: phosphatase and tensin homolog; FGFR: fibroblast growth factor receptor; MVD: microvessel density; AT1R: Angiotensin II Type I Receptor.

*One hundred thirty-one (97.8%) patients were treated with adjuvant intravesical BCG therapy.

**Among 74 patients, 54 (73.0%) were treated with intravesical BCG or MMC therapy.

***Hazard ratio and 95% confidential interval were 0.383 and 0.193–0.961, respectively. *P* value was not shown.

semiquantification of apoptosis may be a useful predictive marker of recurrence after this therapy. The most popular methods of semiquantification of apoptosis in tissue samples are currently TdT-mediated dUTP nick end labeling (TUNEL) and the quantification of caspase-3 levels [39, 40]. These methods were also used in many studies investigating the relationships between apoptosis and carcinogenesis, pathological features, and survival in patients with diseases, including BC [39–41]. However, to our knowledge, only few reports are investigating the prognostic role of the apoptotic index measured by TUNEL and/or caspase-3 expression after intravesical therapy in patients with NMIBC. However, several apoptosis-related molecules were investigated as potential predictive tools and are summarized in Table 1.

4.1. TP53. Wild type p53 is a well-known regulator of cell cycle control in normal cells and acts as a tumor suppressor by controlling apoptotic processes under genotoxic conditions. However, mutations in the p53 gene can induce cell cycle deregulation and apoptosis in cancer cells. Nuclear accumulation of p53 influences the malignant potential and aggressiveness of NMIBC [32, 42]. The prognostic abilities of p53 expression during recurrence after intravesical BCG therapy were investigated, but conflicting results were obtained [17, 19–22, 36, 37, 42–45]. However, as shown in Table 1, the current opinion is that p53 expression has minimal value in predicting recurrence after intravesical BCG therapy. On the other hand, a different study found a mutation in the gene encoding p53 that is associated with the recurrence after intravesical BCG therapy [46]. However, this result was achieved by a univariate analysis and the study included a relatively small sample size ($n = 26$). Thus, although the pathological significance and prognostic role of p53 have been widely investigated, the results of these studies are not conclusive enough to make a clinical decision. On the other hand, in recent years, a meta-analysis demonstrated that p53 overexpression was associated with recurrence-free survival in patients with NMIBC treated with intravesical BCG therapy in the Asian population (hazard ratio = 1.57, 95% confidential interval = 1.08–2.27) [47]. This meta-analysis has several limitations, including presence of heterogeneity, publication bias, and a wide range of cut-off points. However,

we support the opinion that there is value in continuing further studies with rigid criteria and large population.

4.2. p63. The p63 protein is a member of the p53 tumor suppressor protein family [48]. Levels of p63 expression in NMIBC were reported to be higher than those in MIBC [49]. ΔNp63 is an isoform of p63 and it was also reported to act as a tumor suppressor in various cancers [48, 50]. The prognostic potential of p63 and ΔNp63 expression levels was recently investigated in 134 patients with pT1 high-grade cancer (131 of 134 patients (97.8%) that were treated with adjuvant intravesical BCG therapy) [22]. They found that ΔNp63 expression was a significant predictive factor in both univariate and multivariate analyses, whereas p63 expression was not (Table 1). Unfortunately, the molecular mechanisms underlying the prognostic capabilities of ΔNp63 expression are unclear.

4.3. Survivin. Survivin is a member of the inhibitor of apoptosis (IAP) family and it controls various pathological activities by regulating mitosis- and apoptosis-related factors [51]. In patients with BC, survivin expression was associated with malignant potential and recurrence [23, 52]. Univariate and multivariate analyses also demonstrated that survivin expression was a significant and independent predictor of recurrence in 74 patients with NMIBC ($P = 0.003$ and $P = 0.02$, resp.) [23]. Among these 74 patients, however, intravesical therapy was performed in only 54 (73.0%) and the therapies used different agents (BCG or MMC). Although further studies are necessary to conclude the prognostic ability of survivin expression in patients with NMIBC treated with intravesical therapy, this finding facilitates a discussion of the different treatment strategies using intravesical therapy. On the other hand, heterozygous genotypes (GC) of survivin (31G>C) are significantly correlated ($P = 0.0009$) with the recurrence in patients with NMIBC treated with intravesical BCG therapy [24]. These authors also found a considerable relationship with the homozygous genotype (CC) of survivin (31G>C) [24].

4.4. Bcl-2 Family. The apoptotic-related molecules Bax and Bcl-2 are strong pro- and antiapoptotic molecules, respectively. Some studies suggest that these molecules contribute to disease aggressiveness and outcome in BC. However, the opposite results have also been found [32, 37, 53]. A higher Bcl-2/Bax ratio (>1) is associated with early recurrence after adjuvant intravesical chemotherapy [53]. A different report found that Bax expression is significantly associated with the risk of recurrence in intravesical BCG therapy, as determined by univariate analysis ($P = 0.034$) [54]. This study also showed that a model, including pT1 stage, age, and expression levels of Bax and Bcl-2, was the best independent and significant predictor for recurrence after therapy. However, we should note that the study population was relatively small ($n = 28$). As the authors noted, further investigations on a larger cohort are necessary to confidently determine the prognostic roles of Bcl-2 and Bax for intravesical BCG therapy in patients with NMIBC.

5. Angiogenesis and Angiogenesis-Related Molecules

Angiogenesis is a crucial step for tumor growth and progression in almost all types of cancers, including BC [55–58]. In cancer tissues, microvessel density (MVD) is commonly used to evaluate the angiogenic status. Several investigators demonstrated that MVD was significantly associated with the prognosis and recurrence in BC patients [55, 57, 58]. However, the relationship between MVD and recurrence in patients with NMIBC treated with intravesical therapy contradicted previous expectations. MVD was found to be closely associated with recurrence after intravesical BCG therapy as determined by both univariate ($P < 0.0001$, Table 1) and multivariate analyses (hazard ratio, 4.35; 95% confidence interval, 0.90–21.18; $P = 0.0011$) [25]. However, this study population was relatively small ($n = 26$) and the 95% confidence lower limit value was under 1.0. On the other hand, vascular endothelial growth factor (VEGF) promotes angiogenesis in various physiological and pathological conditions. One report found that increased VEGF immunoreactivity is a worse predictor of recurrence in patients with NMIBC treated with intravesical MMC or epirubicin therapy [18]. As for MVD, there is little information regarding the prognostic value of VEGF after intravesical therapy.

6. Other Cancer-Related Molecules

The retinoblastoma protein (pRB) is a well-known tumor suppressor that acts by regulating the cell cycle. One report indicated that pRb expression was not associated with recurrence after intravesical BCG and interferon-alpha therapy ($P = 0.047$) [45]. On the other hand, Cormio et al. [21] reported that altered pRB expression was significantly associated with disease-free survival ($P = 0.037$) in a Kaplan-Meier survival analysis. They also investigated the predicative value of a combined pRb and p53 marker in these patients, but this marker was not significantly associated with survival ($P = 0.08$).

E2F4 is a member of the E2F transcription factor family. E2F4 is involved in cell cycle regulation and the suppression of tumor growth. This protein can bind pRB and there is a report that E2F4 expression level is a useful predictive marker for the effectiveness of intravesical BCG therapy that can predict clinical outcomes, including recurrence, progression, and survival in patients with BC [26]. However, detailed pathological significance and prognostic roles of E2F4 after intravesical therapy are still largely unknown.

Ezrin expression levels are reportedly correlated with cell survival, migration, and adhesion in several cancers [59, 60]. Lower expression levels (<20%) of ezrin in cell membranes are significantly associated with poor disease outcomes in patients with T1 grade 3 disease after intravesical BCG therapy ($P = 0.041$) [34]. Ezrin is a member of the ezrin, radixin, and moesin (ERM) cytoskeleton-associated protein family. This family is composed of ezrin, radixin, and moesin. Currently, the prognostic roles of radixin and moesin in patients with NMIBC are still unknown.

Table 2: Predictive value of immunologic microenvironment and immune cells.

Variable	Patients		Agents	P value for recurrence		Year/reference
	N	Background		Univariate	Multivariate	
CD1a	59	NMIBC	BCG	0.005		2009/[27]
CD1b	59	NMIBC	BCG	<0.000		2009/[27]
CD1c	59	NMIBC	BCG	0.03		2009/[27]
CD1e	59	NMIBC	BCG	0.007		2009/[27]
MHC-1	59	NMIBC	BCG	<0.000		2009/[27]
MIG	59	NMIBC	BCG	<0.0001		2009/[27]
IP10	59	NMIBC	BCG	<0.0001		2009/[27]
TAM	66	NMIBC	BCG	0.101	0.013	2009/[28]
CD68	41	Tis	BCG	0.0002		2009/[29]
TIDC	66	NMIBC	BCG	0.210		2009/[28]
CD83	30	NMIBC	BCG*	0.045	0.039	2009/[28]
Emax	38	Tis	BCG	0.01		2014/[30]
Edgn	38	Tis	BCG	0.04		2014/[30]
EAI	38	Tis	BCG	<0.004		2014/[30]
G/T ratio	38	Tis	BCG	<0.001		2014/[30]
Th2 SB	38	Tis	BCG	<0.0001		2014/[30]

BCG: Bacillus Calmette-Guérin; MHC: major histocompatibility complex; MIG: monokine induced by γ-interferon; IP10: interferon-inducible protein 10 kDa; Emax: eosinophil infiltration; TAM: tumor-associated macrophage; TIDC: tumor infiltrating dendritic cells; Edgn: eosinophil degranulation; EAI: eosinophil activity index; G/T: GATA-3$^+$ lymphocytes/T-Bet$^+$ lymphocytes; Th2 SB: Th2 signature biomarker.
*Patients treated with more than one maintenance BCG cycle.

Mutations in fibroblast growth factor receptor- (FGFR-) 3 have been reported to play important roles in disease progression and outcome in patients with certain malignancies, including BC [61]. However, some reports demonstrated a lack of significant relationship between FGFR-3 mutations and disease recurrence in BC [62, 63]. In addition, Park et al. [19] reported that altered FGFR-3 expression was not associated with recurrence after intravesical BCG therapy.

Phosphatase and tensin homolog (PTEN) plays important roles in tumor suppression via the inhibition of the Akt pathway, and its expression was reported to be decreased in NMIBC [19, 64]. One study investigated the ability of PTEN expression levels to predict recurrence after intravesical therapy in T1 grade 3 tumors, but no significant association was detected [19].

CD9, a motility-related protein, is a member of the transmembrane-4 superfamily. The ability of CD9 to inhibit cell motility has been reported in several cancers. However, there is limited information regarding the pathological roles of CD9 in BC [65]. To our knowledge, only one study has investigated the ability of CD9 expression levels to predict recurrence after intravesical BCG therapy, but no significant association was found ($P = 0.306$) [19].

7. Immune System

The precise mechanisms of the antitumor effects of BCG in the urinary bladder are not fully understood. Many investigators believe that the response to intravesical BCG therapy depends on a patient's ability to generate adequate, massive, and complex immune responses. In addition, a report shows that BCG strains are associated with the clinical impact in NMIBC; 5-year recurrence-free survival rates of patients treated with BCG Connaught are better than those of patients treated with BCG Tice ($P = 0.0108$) [66]. However, other investigators showed no significant difference ($P = 0.896$) between BCG Tokyo and Connaught strains in the 2-year recurrence-free survival rate [67]. On the other hand, regarding immunization against mycobacterial antigens, the maximal peripheral immune response is observed after 4 weekly BCG instillations in patients previously immunized. However, patients not previously immunized required 6 weekly instillations to achieve a maximum stimulation level [68]. Thus, anticancer effects of BCG are regulated by complex immune-related mechanisms.

BCG antigen presentation leads to the regulation of various types of immune cells (e.g., T lymphocytes, natural killer (NK) cells, NKT cells, and macrophages) and changes in the immunologic tumor microenvironment [30, 69]. Anticancer and antibiotic agents can directly affect the immune system by stimulating inflammation. Therefore, in this chapter, we summarize the predictive value of immune-related factors in patients with NMIBC treated with intravesical therapy (Table 2).

7.1. Immunologic Tumor Microenvironment. Nunez-Nateras et al. [30] investigated the relationship between the response to intravesical BCG therapy and pretreatment of the immunologic tumor microenvironment. They investigated the prognostic roles of eosinophil infiltration (Emax) and degranulation (Edgn) and the eosinophil activity index (EAI), which is calculated as Emax + Edgn. They also

investigated the prognostic capabilities of the GATA-3$^+$ Th2-polarized to T-bet$^+$ Th1-polarized lymphocyte (G/T) ratio and the Th2 signature biomarker (Th2 SB), which is calculated as Emax + Edgn + G/T. When these markers were compared between patients that responded to BCG and patients that did not respond to BCG, all markers in responding patients were considerably higher than those in nonresponding patients (Table 2). These authors suggest that the evaluation of Th1 versus Th2 polarization prior to intravesical BCG therapy is useful to predict the response to this therapy. This information is interesting and important to advance our understanding of treatment strategies. However, more detailed and larger studies are necessary to make confident conclusions, because this study had a relatively small ($n = 38$) and nonrandom study population (pTis only).

Another report investigated the relationship between the antitumor effects of intravesical BCG therapy and the expression of antigen-presenting molecules and chemokines [27]. This report showed that patients that did not experience recurrence had increased expression levels of antipresenting molecules and chemokines such as CD1a, CD1b, CD1c, CD1e, major histocompatibility complex- (MHC-) 1, monokine induced by γ-interferon (MIG), and interferon-inducible protein 10 kDa (IP-10), after intravesical BCG therapy.

7.2. Tumor-Associated Macrophage (TAM). The immune response has an impact on the anticancer effects of intravesical BCG therapy. Therefore, many investigators have paid special attention to the prognostic roles of immune cells in BC. Tumor-associated macrophages (TAMs) are associated with malignant aggressiveness and prognosis in various types of cancers, including BC [70, 71]. Several reports have shown that TAM density can predict recurrence in patients with NMIBC [29, 72, 73]. For example, in intravesical BCG therapy, recurrence-free survival in patients with lower numbers of TAMs was better than that of patients with higher TAM counts ($P = 0.0002$) [29]. On the other hand, macrophages exist in two different polarization states classified as M1 and M2. M1 macrophages demonstrate anticancer abilities by promoting various tumor-killing mechanisms, while M2 macrophages suppress cancer-related immune responses, stimulating tumor development and progression [72, 73]. Thus, TAMs in cancer tissues have opposite functions against tumorigenesis and immune activity. Suriano et al. [74] investigated the predictive value of total TAMs as well as M1 and M2 macrophage infiltration after intravesical BCG therapy. Similarly to previous reports [72, 73], they found that total macrophage infiltration is a significant predictor of disease-free survival ($P = 0.020$). In addition, Kaplan-Meier survival analysis showed that a low density of M1-TAM and a high density of M2-TAM were significantly worse predictors of disease-free survival compared to a high density of M1-TAM and a low density of M2-TAM ($P = 0.029$ and $P = 0.02$, resp.) in patients with NMIBC treated with intravesical BCG therapy [74]. These results are interesting and provide critical information about the prognostic roles of TAMs in intravesical BCG therapy. However, the independent roles of M1- and M2-TAMs were not supported by a multivariate analysis.

7.3. Other Immune Cells and Immunity-Related Factors. One study indicated that tumor infiltrating dendritic cell (TIDC) levels, evaluated by CD83 expression, were not associated with recurrence in patients treated with intravesical BCG therapy ($P = 0.210$) [28]. However, when a similar analysis was performed in patients treated with more than one maintenance BCG cycle, TIDC levels were positively associated with the risk of recurrence in both univariate and multivariate analyses ($P = 0.045$ and $P = 0.039$, resp.) [28].

Yutkin et al. [75] investigated the predictive value of NK cell receptor ligands for recurrence after intravesical BCG therapy. Interactions between NK cells and their targets influence natural cytotoxicity receptors under pathological conditions, including cancer. Three natural cytotoxicity receptors (NKp30, NKp44, and NKp46) are currently known. The authors investigated the prognostic roles of these three receptors after intravesical BCG therapy. High expression levels of NKp30, NKp44, and NKp46 were significantly associated with favorable treatment responses ($P = 0.0026$, $P = 0.0027$, and $P = 0.044$, resp.) [75]. However, these results were obtained by univariate analyses only and included a relatively small number of patients ($n = 17$). However, we believe that these findings are critical to understand the immune mechanisms responsible for preventing recurrence in patients with NMIBC treated with intravesical BCG therapy.

In recent years, cytokine panel for response to intravesical therapy (CyPRIT) constructed using urinary levels of inducible cytokines was reported as a potential useful predictor for the risk of recurrence during intravesical BCG therapy [76]. Thus, we agree with the opinion that more detailed and wider analyses of cytokines may lead to the identification of useful predictive factors to guide modifications of the dose and duration of BCG immunotherapy in patients with NMIBC [77].

8. Gene Polymorphism

Polymorphisms of several genes are associated with macrophage susceptibility to intracellular mycobacterial growth, tuberculosis infection, and response to BCG infection [78–80]. *NRAMP1* is one of these macrophage susceptibility-related genes. Several studies investigated the relationships between *NRAMP1* gene polymorphisms and disease outcomes in patients with NMIBC treated with intravesical BCG therapy [81, 82]. Of 22 tumors with the *NRAMP1* D543N G:A genotype, cancer recurred in 4 patients (18.2%). The median time to recurrence was 104.6 months. Of 47 tumors with the *NRAMP1* D543N G:G genotype, cancer recurred in 19 patients (40.4%) and the median time to recurrence was 80.2 months. Thus, the *NRAMP1* D543N G:G genotype was associated with a higher frequency of recurrence and a shorter time to recurrence in patients treated with intravesical BCG therapy ($P = 0.033$). This genotype is recognized as an independent and significant predictive factor for the time to recurrence after intravesical BCG therapy (hazard ratio, 4.57; 95% confidence interval, 1.367–15.272; $P = 0.014$). This study

showed that the *NRAMP1* (GT)n allele 3 was also associated with decreased recurrence time in a similar multivariate analysis model (hazard ratio, 24.789; 95% confidence interval, 3.074–199.883; $P = 0.03$).

The same study also showed that the *hGPX1* CT genotype (Pro-Lue) predicts shorter recurrence times after intravesical BCG therapy ($P = 0.03$) [80]. hGPX1 is a selenium-dependent enzyme and it participates in the detoxification of hydrogen peroxide and oxide radicals. The codon 198 variant of *hGPX1* is reportedly associated with tumor development [83]. A significant relationship between *hGPX* polymorphism and recurrence in patients with NMIBC has also been previously reported [84]. However, this study population included 128 patients treated with intravesical therapy by BCG and 96 patients treated with other agents. Although further studies are necessary to confidently assess the predictive value of *hGPX* polymorphism for outcome after intravesical BCG therapy, we believe that this gene is a likely candidate prognosis marker. In recent years, relationships between genetic variation in glutathione (GSH) pathways and recurrence in patients with NMIBC treated with BCG after TUR were analyzed. This study showed that 7265992 in GSH synthetase was the most significant (hazard ratio = 3.43, 95% confidential interval = 2.19–13.46, $P = 0.0003$) single nucleotide polymorphism in these patients [85].

9. Concluding Remarks

Intravesical therapy is used to prevent recurrence in patients with NMIBC. However, intravesical BCG therapy commonly causes adverse effects. Intravesical therapy with other anticancer agents or antibiotics are relatively safe, but they present lower anticancer effects than BCG. Therefore, predictive markers for anticancer effects, including recurrence prevention, are important tools when selecting treatment strategies for these patients. However, our current understanding of the predictive markers for recurrence after intravesical therapy is insufficient. Despite the fact that many studies have been performed, few results have been verified by multivariate analyses and there is no predictive marker in clinical use. Treatment strategies by intravesical approaches, based on new concepts and ideas, have been recently proposed. Further *in vivo* and *in vitro* studies investigating prognostic markers capable of predicting anticancer effects, clinical outcomes, and adverse effects in new intravesical therapies are required.

Conflict of Interests

The authors declare that there is no potential conflict of interests regarding the publication of this paper.

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Research Article

Association of Cytokeratin and Vimentin Protein in the Genesis of Transitional Cell Carcinoma of Urinary Bladder Patients

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The aim of study was to examine the localization and distribution of cytokeratin (CK) and vimentin protein and their association with clinical outcome of the TCC patients. Expression pattern of cytokeratin and vimentin was evaluated by immunohistochemistry in TCC cases and inflammatory lesions. Cytoplasmic expression of CK was noticed in 52.17% of TCC cases and its expression was not observed in inflammatory lesions of bladder specimens. Vimentin showed expression in 69.00% cases of TCC. Significant differences were noticed in expression pattern of CK and vimentin in inflammatory lesion and Transitional Cell Carcinoma cases. Vimentin expression increased with the grade of TCC and this difference was statistically significant whereas expression of CK decreased according to the grade of TCC. Furthermore, it was also observed that expression pattern of vimentin was high in ≥55 years as compared to <55 age group patients and these differences were significant in men as compared to women. Expression pattern of CK did not show any significant relation with age and gender. Therefore, it can be concluded that cytokeratin and vimentin will be helpful markers in the early diagnosis of Transitional Cell Carcinoma/bladder carcinoma.

1. Introduction

Bladder carcinoma is one of the most common malignancies worldwide in term of morbidity and mortality. Despite of its high prevalence, the molecular mechanism involved in the induction of bladder carcinoma and its progression is not properly understood [1]. Altered expressions of various genes/protein such as tumour suppressor gene, oncogene, and apoptotic genes have been observed in several types of tumour [1, 2]. A range of tumour markers and therapy targets are in use to investigate the Transitional Cell Carcinoma (TCC) and its clinical outcome. But still potential marker is needed to diagnose/investigate the Transitional Cell Carcinoma behaviour. Therefore, the assessment of potential biomarkers will be important move towards diagnosis and treatment of Transitional Cell Carcinoma. However, in this vista, intermediate filament family proteins play an important role in the genesis of Transitional Cell Carcinoma.

Cytokeratins are one of the chief structural proteins, which form the cytoplasmic network of intermediate filaments [3] and its family contains at least 20 types of cytoplasmic intermediate filaments found in epithelial cells [4]. They are expressed in a tissue-specific manner in normal organs as well as in the tumors that derived from them [5]. Different types of expression patterns of cytokeratins were noticed in carcinoma and normal/inflammatory lesions of bladder.

A study report confirmed that 92% of benign/reactive cases were either CK20 (−) or (+) only in the upper 1/3 urothelium whereas in dysplastic cases CK20 staining distribution was noticed as 60% in 2/3 of the urothelium, 30% full thickness, 10% in the upper 1/3 urothelium and among carcinoma in situ (CIS) cases, 89% had full thickness of CK20 positivity [6]. Another valuable study reported that all cases (100%) of normal urothelia had normal expression patterns with Cytokeratin 20 and ninety-six percent of morphologically unequivocal cases of reactive urothelial atypia (RUA) showed normal expression patterns of Cytokeratin 20 whereas, in the carcinoma in situ (CIS) group, 86% had abnormal CK20 expression [7]. Previous finding confirmed that CK20 showed patchy cytoplasmic immunoreactivity in the superficial umbrella cell layer of the normal urothelium [8] and nonneoplastic urothelium showed no reactivity to CK20 except for umbrella cells [9].

Intermediate filaments are one of the three major cytoskeleton networks and these filaments consist of a number of different members such as vimentin and the cytokeratin proteins [10]. Vimentin shows important roles in cell adhesion, migration, and signalling [11]. Numerous studies described the vimentin reactive cells in benign and malignant breast tissues [12, 13] and vimentin expression in the tumour stroma was valuable in identifying colorectal cancer patients with a poor prognosis [14]. Different expression pattern of vimentin was also noticed in bladder cancer and normal urothelia. An important study reported that expression of vimentin was observed in 43% of bladder cancers, whereas it was not expressed or found negative in all normal urothelia [15]. The aim of study was to assess the expression profile of cytokeratin and vimentin in Transitional Cell Carcinoma and its association with clinical outcome such as sex, age, and grade of the tumour via immunohistochemistry.

2. Materials and Methods

2.1. Tissue Specimens. Forty-six patients with Transitional Cell Carcinoma (TCC) and ten cases of inflammatory lesions of bladder, confirmed by histopathologist, were taken to examine the expression profile of both markers and its interpretation with clinical outcome. The patient's details about age and sex were noted as range of 24–78 years with mean age 36 ± 12 years and 38 male and 8 female cases. The TCC cases were further categorized as Grade I ($n = 14$), Grade II ($n = 18$), and Grade III ($n = 14$). Haematoxylin and eosin (H and E) staining was performed on each case to confirm the grading of the tumour.

2.2. Immunohistochemical Detection of Cytokeratin and Vimentin Protein. Transitional Cell Carcinoma (TCC) was sectioned with microtome into 5 μm thick. Deparaffinized was performed with three changes of xylene and blocking of endogenous peroxidase activity was done via 0.3% hydrogen peroxide in methanol for 30 minutes. After the quenching step, antigen retrieval was made with citrate buffer (pH 6.0) in pressure cooker for 30 minutes and then sections were kept at room temperature for 20–30 minutes. Then, blocking step was made with blocking agent for 10 minutes and sections were washed with PBS. Monoclonal mouse anti-human cytokeratin (Clone AE1/AE3, Dako) and Monoclonal mouse Anti-Vimentin (Clone V9, Dako) antibodies was applied at 1 : 100 and 1 : 75 dilutions for 2 hours at room temperature in humid chamber. Following incubation with secondary antibody for 45 minutes, followed by incubation with streptavidin-biotin enzyme complex was applied for 30 minutes. Finally, diaminobenzidine (DAB) chromogen was used and then sections were counterstained with haematoxylin.

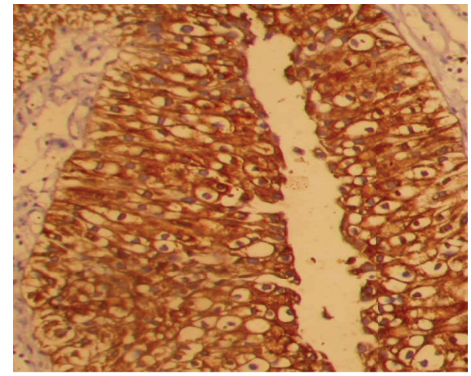

Figure 1: Cytoplasmic expression of cytokeratin protein in Transitional Cell Carcinoma (orig. mag ×100).

2.3. Evaluation of Staining. Negative (omission of antibody) and positive controls (oral cancer) cases were run to verify the quality of staining and confirmation of the procedures. Markers such as cytokeratin and vimentin were considered as positive if more than 5% of cells were positive and less than 5% cytoplasmic positivity was taken as negative. Cytoplasmic expression of cytokeratin and vimentin was considered as positive cases. All fields of the section were analyzed by two pathologists and more than 400 tumor cells, in five different area, were counted and mean percentage was calculated.

2.4. Statistical Analysis. Markers' expression and its association with clinical outcome were analyzed by Chi square $(\lambda)^2$. The P value $P < 0.05$ was considered as statistically significant.

3. Results

Cytokeratin and vimentin markers were analyzed according to age, sex, and histological grade and results were interpreted accordingly.

3.1. Immunohistochemical Analysis of Cytokeratin Protein. Cytokeratin expression was noticed in 24.00 (52.17%) of TCC cases in cytoplasm (Figure 1) including 9 (64.28%) in Grade I, 10 (55.55%) in Grade II, and 5 (35.71%) in Grade III whereas 22.00 (47.82%) of TCC did not show any expressions of cytokeratin protein (Figure 2). Furthermore, the expression profile was examined according to age and sex and difference of expression pattern was insignificant

Table 1: Expression of cytokeratin (CK) and vimentin in TCC and inflammatory lesion cases and its association with grade of the tumour.

Clinical parameters patients	CK expression			Vimentin expression		
	Total cases	% positivity	*P* value	Positive cases	% positivity	*P* value
Inflammatory lesions	10	00		01	10.00	
Tumor grades						
I	9	64.28		6	42.85	
II	10	55.55	<0.05	13	72.22	<0.05
III	5	35.71		13	92.85	
Total cases	24	52.17		32	69.56	
Sex						
Male	20	52.60	>0.05	31	81.00	<0.05
Female	04	50.00		01	12.5	
Age						
<55 years	8	50.00	>0.05	7	43.00	
≥55 years	16	55.00		25	63.3	

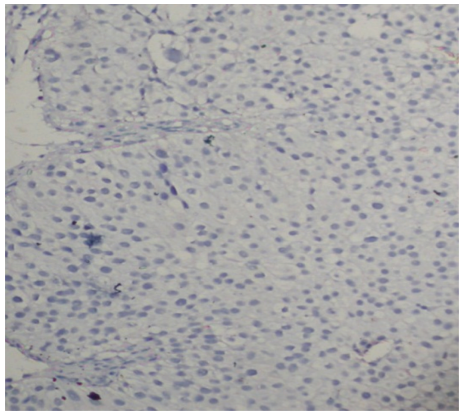

Figure 2: Cytokeratin did not show expression in Transitional Cell Carcinoma (orig. mag ×100).

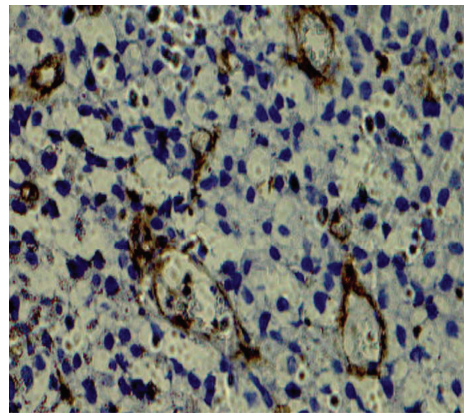

Figure 3: Vimentin showed expression in Transitional Cell Carcinoma (orig. mag ×100).

(Table 1). Markedly, the positivity of CK decreased according to grade of the tumour, but this difference was statistically significant. Among inflammatory lesions of bladder cases, CK did not exhibit any expression. The expression pattern of CK was measured in both sexes and less than 50 years and equal to or more than 50 years age group, but differences in expression pattern of CK did not reach statistically significant level in gender and different age groups.

3.2. Positivity of Vimentin in TCC and Inflammatory Lesions of Bladder Cases. Vimentin expressions were analyzed in TCC cases and it was noticed that vimentin was overexpressed in 32 (69.56%) of TCC cases (Table 1 and Figure 3) and 14 cases (30.43%) did not show any expression of vimentin (Figure 4). One case out of 10 showed expression in inflammatory lesions of bladder. This difference of expression pattern in TCC and inflammatory lesions was statistically significant. Expression of vimentin was further categorized according to grade, gender, and sex. The positivity of vimentin increased according to grade of the TCC and it was (6 cases, 42.85%), (13 cases, 72.22%), and (13 cases, 92.85%) in Grade I, Grade II and Grade III, respectively (Table 1). The expression pattern

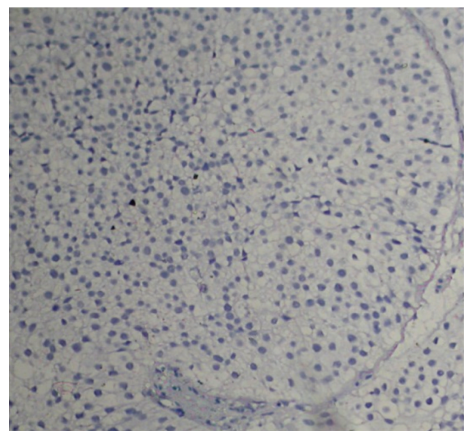

Figure 4: Vimentin did not show expression in Transitional Cell Carcinoma (orig. mag ×100).

of vimentin was statistically significant according to grade. The TCC cases were divided into two groups on the basis of age, that is, <55 years and ≥55 years, and it was observed that expression of vimentin was high in ≥55 years as compared

to <55 years and these differences were significant in men as compared to women (Table 1).

3.3. Correlation of Both Markers and Their Interpretation according to Age and Gender. Transitional Cell Carcinoma (TCC) cases were examined for both markers; most of the cases showed both CK and vimentin positivity and our results showed that cytokeratin and vimentin have pivotal role in development and progression of TCC. A negative correlation was observed in CK and vimentin expression with gradewise observations. Markedly, the positivity of CK decreased according to grade of TCC whereas vimentin increases according to the grade of the carcinoma.

4. Discussion

Bladder cancer is the fifth most common cancer worldwide and also one of the major causes of cancer morbidity and mortality [16]. It affects men more as compared to women (3 : 4.1) [17]. Our finding also showed similar pattern and most of TCC patients were male (38 cases, 82.60%) and (8 cases, 17.39%) were female. The exact reason behind this is not understood, but it is thought that males acquire smoking, chewing, and drugs habits earlier than women. Currently, various markers are in practice to diagnose the Transitional Cell Carcinoma via immunohistochemistry. But still a potential marker is needed to diagnosis the early bladder carcinoma/Transitional Cell Carcinoma. In this vista, our study tried to find some more information of CK and vimentin role in the diagnosis of bladder carcinoma/Transitional Cell Carcinoma.

In our finding expression of cytokeratin was noticed in 24 cases of TCC (52.17%) and CK was not expressed in inflammatory lesion of bladder cases. Furthermore, our results showed that the expression pattern of CK decreased according to the grade of the TCC (from Grades I to III) and these differences were statistically significant. Several findings showed different types of results in this issue and some results were in accordance with our study. One of the study results revealed that positivity of Cytokeratin 20 associated with increasing tumor grade and stage and it was observed that 69.4% cases of high grade tumors showed Cytokeratin 20 positivity as compared to 45.00% of other grades of tumours [18]. Another study reported that CK-20 expression was observed in all grades of tumour as 75.00% of low malignancy potential, 83.00% of low grade, 38.00% of high grade, and 67.00% of high grade tumors that invaded adjacent structures [19]. Other important study results showed that reduction or loss of cytokeratin expression was significantly correlated with tumor stage and grade [20]. Our finding did not reach significant level in terms of positivity of CK in gender and age basis analysis.

In the current study vimentin expressions were only noticed in cytoplasm and 32 cases (69.56%) of TCC showed expression of vimentin. Previous finding also showed high expression in Transitional Cell Carcinoma cases. A study finding showed that 30 (24.8%) were positive for vimentin in bladder cancer patients [21] and other important investigations showed that 43.00% bladder cancer expressed vimentin [15].

The present study results confirmed that vimentin expression is associated with the grade of the tumour. A recent study showed that expression of HMGA2, loss of E-cadherin, and expression of vimentin are significantly correlated with bladder cancer grade and stage [15]. Another study also confirms that vimentin was found to have statistically significant correlations with grade, recurrence, and progression [21]. Our results show that expression was different in different age group and it was noticed that older age group (≥55) showed high expression (63.00%) of vimentin especially in male. The exact reason is difficulty to explain the difference in expression pattern of both markers in cancer and inflammatory lesions. But it is thought that long cumulative exposure of carcinogen plays a critical role in the DNA damage/DNA adduct formation and alterations in transitional epithelial. The current study demonstrates that significant difference of expression pattern of both markers in TCC and inflammatory lesions of bladder and expression of vimentin was closely associated with the grade of TCC. Keeping in view the above information, our study concluded that use of both markers will be helpful in the diagnosis/investigation of early Transitional Cell Carcinoma.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

CD73 Predicts Favorable Prognosis in Patients with Nonmuscle-Invasive Urothelial Bladder Cancer

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Aims. CD73 is a membrane associated 5′-ectonucleotidase that has been proposed as prognostic biomarker in various solid tumors. The aim of this study is to evaluate CD73 expression in a cohort of patients with primary bladder cancer in regard to its association with clinicopathological features and disease course. *Methods*. Tissue samples from 174 patients with a primary urothelial carcinoma were immunohistochemically assessed on a tissue microarray. Associations between CD73 expression and retrospectively obtained clinicopathological data were evaluated by contingency analysis. Survival analysis was performed to investigate the predictive value of CD73 within the subgroup of pTa and pT1 tumors in regard to progression-free survival (PFS). *Results*. High CD73 expression was found in 46 (26.4%) patients and was significantly associated with lower stage, lower grade, less adjacent carcinoma in situ and with lower Ki-67 proliferation index. High CD73 immunoreactivity in the subgroup of pTa and pT1 tumors ($n = 158$) was significantly associated with longer PFS (HR: 0.228; $p = 0.047$) in univariable Cox regression analysis. *Conclusion*. High CD73 immunoreactivity was associated with favorable clinicopathological features. Furthermore, it predicts better outcome in the subgroup of pTa and pT1 tumors and may thus serve as additional tool for the selection of patients with favorable prognosis.

1. Introduction

Bladder cancer (BC)—the 11th most commonly diagnosed cancer worldwide [1]—presents in up to 80% of all patients either as a noninvasive papillary carcinoma (pTa) or as a carcinoma invading the submucosal connective tissue (pT1) [2, 3]. However, approximately 70% of these superficial tumors recur and up to a quarter even progress into muscle invasive bladder cancer (MIBC) [4]. Since close cystoscopic monitoring of recurrence and progression after initial transurethral resection of the tumor causes immense healthcare costs [5], accurate markers are needed in addition to clinicopathological factors [6] to individualize postoperative follow-up schedules [7–9]. Although numerous studies have evaluated the predictive value of different biomarkers in regard to progression of superficial bladder cancer [10–19], none of them has an established role in daily clinical practice.

CD73 (NT5E, ecto-5′-nucleotidase) is a glycosylphosphatidylinositol- (GPI-) anchored cell-surface enzyme that plays a crucial role in the purinergic signalling pathway by dephosphorylating AMP (adenosine monophosphate) into adenosine [20, 21]. Extracellular adenosine itself is involved in tumor immunoescape and invasion of tumor cells [22], while nonenzymatic functions of CD73 are related to cell adhesion and migration of tumor cells [23, 24].

CD73 expression has been investigated in many different cancer cell lines and human tumor biopsies so far and seems to play an important role in cancer development [20, 25, 26]. The role of CD73 in bladder BC is not well known and controversial [27–30]. Furthermore, neither larger expression studies of CD73 in BC biopsies nor studies investigating the predictive ability of this marker have been published so far. The aim of the present study is to evaluate the association

Table 1: Patient and tumor characteristics and results of immunohistochemical analyses.

Variable	Categorization	n analyzable[a]	%
	Total (n = 174)[a]		
	Clinicopathologic data		
Age at diagnosis (median, range): 69.5 years (32–92)	<70 years	87	50.0
	≥70 years	87	50.0
Sex	Female	43	24.7
	Male	131	75.3
Tumor stage (WHO 1973[b])	pTa	90	51.7
	pT1	68	39.1
	pT2	13	7.5
	pT3	2	1.1
	pT4	1	0.6
Histologic grade (WHO 1973[b])	G1	44	25.3
	G2	87	50.0
	G3	43	24.7
Histologic grade (WHO 2004[c])	Low grade	101	58.0
	High grade	73	42.0
Adjacent carcinoma in situ	No	158	90.8
	Yes	16	9.2
Multiplicity	Solitary	124	71.3
	Multifocal	50	28.7
Growth pattern	Papillary	159	91.4
	Solid	15	8.6
	Immunohistochemistry (IHC)		
CD73	Score 0	80	46.0
	Score 1+	48	27.6
	Score 2+	46	26.4
Ki-67 labelling index	≤10%	108	62.1
	>10%	66	37.9

[a]All patients.
[b]Staging and grading according to the 1973 WHO classification system.
[c]Staging and grading according to the 2004 WHO classification system.

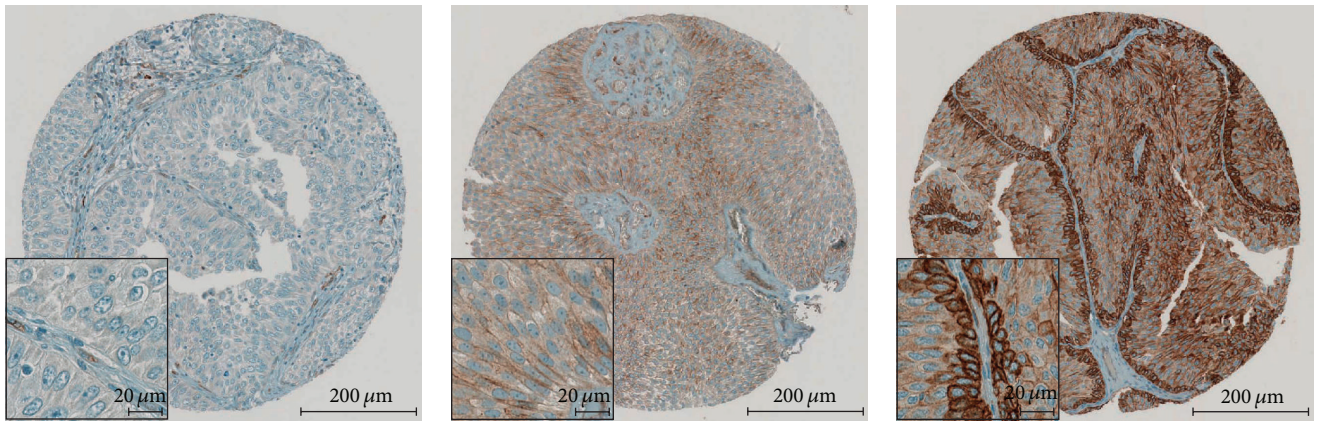
between CD73 expression and tumor progression in a large cohort of patients with primary BC.

2. Material and Methods

Tissue microarrays (TMA) were constructed from 348 formalin-fixed, paraffin-embedded urothelial BC tissues from 174 patients as previously described [31]. Tumor samples were represented in duplicate tissue cores with a diameter of 1 mm. The collection of the specimens was performed by the Institute of Surgical Pathology, University Hospital, Zurich, Switzerland, between 1990 and 2006. The tissue samples in TMA represent a series of 174 consecutive (nonselected) primary urothelial bladder tumors consisting of 90 pTa, 68 pT1, and 16 ≥ pT2 tumors. A board-certified pathologist (Peter Wild) reevaluated the hematoxylin-and-eosin-stained slides of all specimens. Tumor stage and grade were assigned according to UICC and WHO criteria. Additionally, to analyse the immunoreactivity of CD73 in normal urothelium, eight slides were cut from formalin-fixed, paraffin-embedded urothelium of the bladder neck of patients without any history of urothelial dysplasia or BC.

Retrospective clinical follow-up data were available for all the 174 patients (100%). The median follow-up period for the entire cohort was 110.6 months ranging from 32.4 to 226.8 months. Unfortunately, a proper analysis of adjuvant bladder instillation therapy (BCG or chemotherapy) could not be performed due to missing data in about 50% of the patients. The TMA and its clinical cohort have been previously published [32]. Descriptive characteristics of the cohort are depicted in Table 1. The study was approved by the Cantonal Scientific Ethics Committee Zurich (http://www.kek.zh.ch, approval number: StV-NR. 25/2007).

TMA was freshly cut and used on 3 μm paraffin sections as described previously [33]. The immunohistochemical detection of CD73 on tissue samples was performed by use of Anti-NT5E rabbit polyclonal antibody from Sigma Chemical Company, Saint Louis, United States (dilution



(a) (b) (c)

Figure 1: Immunohistochemical staining pattern of CD73. CD73 shows a distinct membranous immunoreactivity with pronounced intensity in the basal layer. Representative examples of score 0 (a), 1+ (b), and 2+ (c) are depicted.

1 : 500). Clone MIB-1 (dilution 1 : 50; Dako, Glostrup, Denmark) was used for the detection of Ki-67. Immunohistochemical studies utilised an avidin-biotin peroxidase method with a diaminobenzidine (DAB) chromogen. After antigen retrieval (microwave oven for 30 min at 250 W), immunohistochemistry was conducted using a NEXES autostainer (Ventana, Tucson, AZ, USA) following the manufacturer's instructions.

Two experienced pathologists (Lorenz Buser and Peter Wild) evaluated all slides. Membranous CD73 immunoreactivity in the basal layer was assessed by using a semiquantitative three-scale scoring system ranging from 0 to 2+ (score 0: no staining; score 1+: weak staining; score 2+: strong staining).

In the situation of observing different staining intensities between the duplicate tissue cores, the core with more representative tumor tissue was chosen. If both duplicate tissue cores with different staining intensities demonstrate a comparable amount of representative tumor tissue, the intensity of the core with more homogenous staining intensity was selected. Immunoreactivity of CD73 was dichotomized for analytical purposes into a CD73 low-expression group (containing scores 0 and 1+) and a CD73 high-expression group (containing score 2+). The percentage of Ki-67 positive cells of each specimen was determined as described previously [34]. More than 10% of positive tumor cells were defined as a high Ki-67 labelling index [35].

Statistical analyses were performed with SPSS version 22 (IBM, Armonk, USA). p values < 0.05 were considered as statistically significant. The statistical associations between clinicopathological and immunohistochemical data were analysed by using contingency tables together with Fisher's exact test (2-sided) for all binary variables and the Chi-square test (2-sided) for all other nominal variables. In the subgroup of nonmuscle-invasive bladder cancer (NMIBC, pTa and pT1), progression-free survival (PFS) was evaluated. Stage-shifts (from pTa to pT1-4 or from pT1 to pT2-4) or the detection of distant metastasis was considered as progression. The Kaplan-Meier method was used for plotting the PFS curves. Significant differences between the curves were analysed by using the two-sided log-rank test. Associations between clinicopathological/immunohistochemical data and PFS were evaluated by univariable and multivariable Cox regression. All significant variables in the univariable analysis were used in the multivariable model.

3. Results

All 174 (100%) tumor samples could be evaluated for CD73 immunoreactivity. CD73 showed a membranous immunoreactivity, which was much more pronounced in the basal layer than in the other tumor cells. Further, a faint and inhomogeneous cytoplasmatic staining was observed in most of the cases with positive membranous staining. In order to allow a distinct evaluation, only membranous CD73 immunoreactivity in the basal layer was assessed. Figure 1 shows representative examples from our TMA for each staining score ranging from 0 to 2+.

A strong expression of CD73 (score 2+) was found in 46 of 174 patients (26.4%), whereas in 48 patients (27.6%) a weak immunoreactivity (score 1+) was detected. Finally, 80 patients (46%) showed no CD73 staining. The distribution of the dichotomized staining intensities of CD73 and Ki-67 are shown in Table 1. Additionally, we investigated 8 samples of normal urothelium (Supplementary Figures S1A–F in Supplementary Material available online at http://dx.doi.org/10.1155/2015/785461) of individuals without any history of urothelial dysplasia or carcinoma. We observed a weak and inhomogeneous expression of CD73 in the cytoplasm and cell membrane, which was more pronounced in the basal layer in 4 out of 6 cases (Supplementary Figures S1A–C, F).

Table 2 shows the associations between CD73 staining patterns and clinicopathological parameters (stage, grade, adjacent carcinoma in situ, multiplicity, growth pattern, and Ki-67) of the tumors. High CD73 expression is associated with lower stage ($p = 0.006$), lower grade (WHO 2004,

Table 2: Comparison of the immunohistochemical markers with pathologic characteristics ($n = 174$).

Variable	Categorization	CD73 expression		p
		Score 0 or 1+	Score 2+	
Tumor stage (WHO 1973)[a]	pTa	57	33	**0.006**
	pT1	57	11	
	pT2	12	1	
	pT3	2	0	
	pT4	0	1	
Histologic grade (WHO 1973)[a]	G1	31	13	0.100
	G2	60	27	
	G3	37	6	
Histologic grade (WHO 2004)[b]	Low grade	67	34	**0.014**
	High grade	61	12	
Adjacent carcinoma in situ[b]	No	112	46	**0.007**
	Yes	16	0	
Multiplicity[b]	Solitary	87	37	0.130
	Multifocal	41	9	
Growth pattern[b]	Papillary	115	44	0.359
	Solid	13	2	
	Immunohistochemistry			
Ki-67 labelling index	≤10%	72	36	**0.008**
	>10%	56	10	

[a]Chi-square Pearson (2-sided); bold face representing p values <0.05.
[b]Fisher's exact test (2-sided); bold face representing p values <0.05.

$p = 0.014$), less adjacent carcinoma in situ ($p = 0.007$) and lower Ki-67 expression ($p = 0.008$). Contingency table analysis for the Ki-67 labelling index and the clinicopathological characteristics have been previously published [32] and showed a significant correlation ($p < 0.05$) with all clinicopathological characteristics except for tumor multiplicity.

158 out of 174 patients (90.8%) underwent TUR for a primary pTa or pT1 urothelial carcinoma of the bladder. This subgroup was followed for a median of 110.7 months (range: 32.4–245.9 months). In total, 22 patients (13.9%) showed a stage-shift. The median time to progression was 45.2 months ranging from 10.2 to 226.7 months. Kaplan-Meier analysis shows that patients within the subgroup of low CD73 expression have a significantly shorter PFS compared to the subgroup of high CD73 expression (Figure 2). The corresponding log-rank test renders a p value of 0.030. Growth pattern ($p < 0.001$) and Ki-67 ($p = 0.003$) were also significantly associated with shorter PFS (Table 3).

In univariable Cox regression (Table 4), a high expression of CD73 reduces the rate of progression (hazard ratio, HR) by the factor of 0.228 (95% confidence interval [CI] ranging from 0.053 to 0.978; $p = 0.047$) compared to patients with a low expression of CD73. Of all other clinicopathologic parameters, growth pattern (HR = 7.634 [2.717–21.740]; $p < 0.001$) and Ki-67 (HR = 3.356 [1.429–7.874]; $p = 0.006$) were significantly associated with reduced PFS. We performed multivariable Cox regression analysis for all significant predictors in univariable analysis, where CD73 did not remain a significant predictor. Only growth pattern remained an independent predictor for shorter PFS (HR = 3.891 [1.245–12.195]; $p = 0.019$).

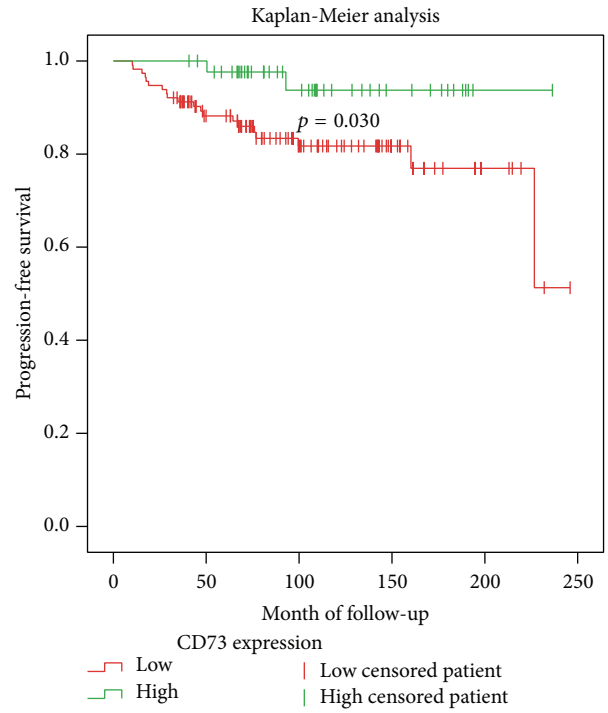


Figure 2: Kaplan-Meier analysis for progression-free survival comparing high and low CD73 expression. The log-rank test was used for the detection of statistical significance.

Table 3: Analysis of factors for tumor progression.

Variable	Categorization	Tumor progression (TP)		
		n^a	Events	p^b
	Pathologic data			
Tumor stage (WHO 1973[c])	pTa	90	10	0.360
	pT1	68	12	
Histologic grade (WHO 1973[c])	G1	44	3	0.085
	G2	86	12	
	G3	28	7	
Histologic grade (WHO 2004[d])	Low grade	99	10	0.083
	High grade	59	12	
Adjacent carcinoma in situ	No	146	20	0.545
	Yes	12	2	
Multifocality	Unifocal tumor	115	15	0.465
	Multifocal tumor	43	7	
Growth pattern	Papillary	151	17	**<0.0001**
	Solid	7	5	
	Immunohistochemistry			
CD73	Score 0 or 1+	114	20	**0.030**
	Score 2+	44	2	
Ki-67 labelling index	≤10%	106	9	**0.003**
	>10%	52	13	

[a]Only primary pTa and pT1 tumors are included.
[b]Log-rank test (2-sided); bold face representing p values <0.05.
[c]Staging and grading according to the 1973 WHO classification system.
[d]Staging and grading according to the 2004 WHO classification system.

Table 4: Regression analysis.

Variable (categorization)	Univariable analysis				Multivariable analysis			
	HR	95% CI		p value	HR	95% CI		p value
Tumor stage (pTa versus pT1)	1.481	0.635	3.454	0.363				
Histologic grade (WHO 2004[a])	2.070	0.894	4.808	0.090				
Adjacent carcinoma in situ	1.563	0.363	6.711	0.549				
Multifocality	1.401	0.565	3.472	0.467				
Growth pattern (papillary versus solid)	7.634	2.717	21.740	**<0.001**	3.891	1.245	12.195	**0.019**
CD73	0.228	0.053	0.978	**0.047**	0.319	0.072	1.408	0.132
Ki-67	3.356	1.429	7.874	**0.006**	2.222	0.873	5.650	0.094

[a]Staging and grading according to the 2004 WHO classification system.

4. Discussion

This is the first study evaluating CD73 immunoreactivity in a large cohort of primary urothelial bladder carcinomas. We found that high CD73 expression is associated with lower stage, lower grade (WHO 2004), less adjacent carcinoma in situ and lower Ki-67 expression. In the subgroup of NMIBC (pTa and pT1), high CD73 immunoreactivity was associated with longer PFS in univariable Cox regression analysis but did not remain an independent predictor of longer PFS in multivariable Cox regression analysis.

Only a few studies have evaluated the role of CD73 in BC. Similar findings were reported by Wilson et al. who studied neoplastic transformed rodent bladders and detected a loss of CD73 after cancerous transformation [29]. A study using two human bladder cancer cell lines found a CD73 activity in the higher grade cell line of about five times as high as in the lower grade cell line [27]. Rockenbach et al. induced bladder cancer in mice and detected a higher expression of CD73 in the cancerous tissue [30]. One study evaluated CD73 enzyme activity in 36 human bladder cancer biopsies and 9 noncancerous bladder biopsies [28]. CD73 enzyme

activity was comparable in BC and in normal urothelial tissue. However, contrary to our work, no follow-up data was reported on BC patients.

Several studies have investigated CD73 immunohistochemistry in other solid tumors. Interestingly, similar results have been reported for gynaecologic neoplasias: Oh et al. investigated 167 epithelial ovarian carcinomas and found associations between overexpression of CD73 and better prognosis, lower stage, and better differentiation [36]. Another study also assigning a favorable prognosis to elevated CD73 expression is the one of Supernat et al. that analysed 136 breast cancers (stages I–III) [37].

However, other investigations of CD73 in different solid tumors found contradictory results: CD73 has been reported as a disadvantageous prognostic or predictive factor, namely, in colorectal, gastric, gallbladder, prostate, and some forms of breast cancer [38–43].

Taken together, conflicting results have been published for the role of CD73 in solid tumors. Interestingly CD73 can be upregulated by several different mediators and conditions such as hypoxia [44, 45] and IFN-beta [46]. Hypoxia is a common characteristic of advanced cancers with poor progression while an increase in IFN-beta activates immunity against tumors [47]. Presence of hypoxia or IFN-beta in these tumors could partially explain the discrepancies. Furthermore, it is important to note that different tissues have very different enzymatic activity of CD73 [48]. This as well can potentially explain differences between different cancers.

In conclusion, our results are in contrast with the current proposed concepts from some authors, where overexpression of CD73 is considered as a disadvantageous factor in carcinogenesis [20, 25, 26]. In our study strong CD73 expression was associated with low-grade tumors, known for their good prognosis. Similar to our work, Oh and Rackley et al. could also detect firstly an inverse relationship between grading and expression of CD73 in epithelial ovarian carcinomas and prostate cancers, respectively, and secondly a direct relationship between expression of CD73 and advantageous prognosis [36, 49]. As in our work, strong CD73 expression was also proposed as a potential marker of good prognosis in breast cancer [37]. Very recently, a molecular biologic link between BC and breast cancer has been found, where a study identified two intrinsic, molecular subsets of high-grade BC, which have similar characteristics of subtypes of breast cancer [50]. The results of this work suggest that molecular characteristics of BC reflect many aspects of breast cancer. Since CD73 promotes the hydrolysis of AMP into adenosine and phosphate [20, 21], the results of our study could also be explained by the proapoptotic effect of adenosine postulated by several articles [51–53].

In our study, high CD73 expression is associated with lower stage and grade and therefore with low risk disease. However, we note that low CD73 expression is also present in a distinct amount of patients with pTa tumors ($n = 57$, 33%; see Table 2). According to our results presented in Table 2, it seems that strong expression of CD73 has good specificity (0.85) but low sensitivity (0.63) in the prediction of low risk (pTa) tumors.

There are limitations to our study. First, this is a retrospective, single-institution study. Second, information about adjuvant instillation therapy could not be evaluated. As tumor recurrence and progression is known to vary upon instillation therapy, it is an important missing aspect of this study. Also, the investigated study population has been collected over a long time period between 1990 and 2006 where clinical practice with respect to intravesical therapy has changed. Thus, this may have additionally influenced our study results. However, all patients had primary (nonrecurrent) tumors and a long follow-up period, which makes this study population nevertheless meaningful to study. Third, the low rate of MIBC patients has to be mentioned. Hence, no conclusion can be drawn for this group of tumors. Prospective studies with a higher proportion of MIBC are necessary to further evaluate the role of CD73 as a biomarker in BC. Furthermore—beside validation studies for the function of CD73 as a biomarker in BC—more research investigating the role of CD73 as a possible therapeutic target is warranted.

5. Conclusion

High expression of CD73 is observed in BC patients with favorable pathological features. In NMIBC, high CD73 expression was additionally associated with good prognosis. Besides clinical and pathological parameters, detection of strong CD73 expression in BC may help to stratify patients into a low risk group. Further studies are warranted to confirm this hypothesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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