Expression Signature Defined by FOXM1–CCNB1 Activation Predicts Disease Recurrence in Non–Muscle-Invasive Bladder Cancer

Seon-Kyu Kim1, Yun-Gil Roh2, Kiejung Park1, Tae-Hong Kang2, Wun-Jae Kim3, Ju-Seog Lee4, Sun-Hee Leem2, and In-Sun Chu1

Abstract

Purpose: Although standard treatment with transurethral resection and intravesical therapy (IVT) is known to be effective to address the clinical behavior of non–muscle-invasive bladder cancer (NMIBC), many patients fail to respond to the treatment and frequently experience disease recurrence. Here, we aim to identify a prognostic molecular signature that predicts the NMIBC heterogeneity and response to IVT.

Experimental Design: We analyzed the genomic profiles of 102 patients with NMIBC to identify a signature associated with disease recurrence. The validity of the signature was verified in three independent patient cohorts (n = 658). Various statistical methods, including a leave-one-out cross-validation and multivariate Cox regression analyses, were applied to identify a signature. We confirmed an association between the signature and tumor aggressiveness with experimental assays using bladder cancer cell lines.

Results: Gene expression profiling in 102 patients with NMIBC identified a CCNB1 signature associated with disease recurrence, which was validated in another three independent cohorts of 658 patients. The CCNB1 signature was shown to be an independent risk factor by a multivariate analysis and subset stratification according to stage and grade [HR, 2.93; 95% confidence intervals (CI), 1.302–6.594; P = 0.009]. The subset analysis also revealed that the signature could identify patients who would benefit from IVT. Finally, gene network analyses and experimental assays indicated that NMIBC recurrence could be mediated by FOXM1–CCNB1–Fanconi anemia pathways.

Conclusions: The CCNB1 signature represents a promising diagnostic tool to identify patients with NMIBC who have a high risk of recurrence and to predict response to IVT. Clin Cancer Res; 20(12); 3233–43.

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Introduction

Bladder cancer is the sixth most prevalent type of cancer worldwide and is responsible for the deaths of 150,000 individuals annually (1). Urothelial carcinoma of the bladder represents more than 90% of all bladder cancers, approximately 80% of which are non–muscle-invasive bladder cancer (stage Ta or T1; NMIBC). However, patients with NMIBC experience frequent relapse of the disease after treatment, and approximately 20% progress to muscle-invasive bladder cancer (Stages T2, T3, or T4; MIBC). Although conventional clinical variables, such as the stage, grade, tumor size, number of tumors, and presence of concomitant carcinoma in situ (CIS), are generally considered to be prognostic factors, the usefulness of these factors to predict patient outcomes is limited (2). Intravesical therapy (IVT) of Bacillus Calmette–Guérin (BCG) combined with transurethral resection (TUR) of bladder cancer is recognized as the best treatment option for the prevention or delay of recurrence or progression in high-risk NMIBC (2–4). However, many patients fail to respond to BCG therapy and are at a high risk of disease recurrence and progression (3–5).

Recent genome-wide gene expression studies in bladder cancer strongly indicate that tumor heterogeneity is well reflected in gene expression patterns (6–11). Furthermore, genome-wide gene expression profiling revealed that a predictive signature for the response to IVT could be identified (12). Indeed, a number of genome-wide studies have been conducted on NMIBC, yet there are no reliable criteria...
that can adequately predict disease recurrence in NMIBC. Moreover, although numerous clinical investigations associated with IVT, including genome-wide approaches, have been performed, their predictive ability of the response to IVT remains insufficient or the relevant patients with cancer included were limited (3, 5, 12–15).

Here, we investigate putative genetic signatures associated with disease recurrence in NMIBC using multiple patient cohorts. To explore all possible disease-driving genes and interactive gene sets, we applied a genome-wide survey of gene expression data based on an iterative in trans correlation approach and attempted to distinguish subgroups of NMIBC that have distinct biologic characteristics associated with NMIBC recurrence. To validate the utility of the signature, we further attempted to test whether the newly identified gene set signature could identify patients with NMIBC who had a significant benefit from IVT. Through a number of experimental assays, we also verified a strong association between the signature and NMIBC aggressiveness using bladder cancer cell lines.

Materials and Methods

Patients and gene expression data

We used previously published clinical and gene expression data from 165 primary bladder cancer patients (9). Briefly, tissue samples from 165 patients with histologically diagnosed urothelial carcinoma were obtained from the Chungbuk National University Hospital, Cheongju, South Korea. The collection and analysis of all the samples was approved by the institutional review board of Chungbuk National University, and informed consent was obtained from each subject. Among the 165 patient samples, we used 102 primary NMIBC samples as the exploration dataset (the Korean cohort, n = 102). All of the gene expression data are available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) public database under the data series accession number GSE13507. Another gene expression dataset from a European consortium for bladder cancer microarray study (GSE5479, n = 404) was used for validation of the signature (7). Among these, 353 NMIBC samples were selected as the first validation dataset for the current study (the European cohort, n = 353). Two other gene expression datasets of patients with NMIBC from hospitals of the Swedish southeastern healthcare region (GSE32894; the SSH cohort, n = 213; ref. 16) and Skane University Hospital (GSE32549; the SUH cohort, n = 92; ref. 17) were used as the second validation datasets for confirmation of the gene expression signature.

In all four patient cohorts, the vast majority of patients with NMIBC received complete TUR and subsequent adjuvant IVT with BCG or mitomycin-C (MMC). Of 760 patients with NMIBC, 136 (71 in stage Ta, 65 in stage T1) had received IVT; the remaining patients did not receive IVT (n = 319), or the treatment data were not applicable (n = 305). Disease recurrence was defined as the relapse of primary NMIBC at a lower or equivalent pathologic stage (Ta or T1). Table 1 details the baseline characteristics of the patients in all cohorts.

Iterative in trans correlation analysis

To generate an in trans gene set highly associated with a gene feature, we applied a Pearson correlation test to the gene expression data from the Korean cohort and selected the genes having significant correlation coefficients with an initial gene feature across patients with NMIBC (|r| > 0.4 and P < 0.001). Using a gene expression data matrix consisting of a gene feature and its correlated genes, we performed a hierarchical clustering analysis with the centered correlation coefficient as the measure of similarity and complete linkage clustering method. According to the patient clustering result, the patients were divided into two subgroups, and the time to recurrence of the patients in each subgroup was estimated. The Kaplan–Meier method was used to calculate the time to recurrence-free survival, and differences in survival between the two subgroups are assessed using log-rank statistics. To estimate the prognostic values of all gene features with their correlated gene sets, a Pearson correlation test, hierarchical clustering, a Kaplan–Meier analysis, and a log-rank test were repeatedly applied to all gene features existing in the gene expression data from the Korean cohort. A P value < 0.01 by log-rank test and number of correlated genes >1,000 were considered statistically significant.

Validation procedure

For validation of the prognostic value of the molecular signature, we developed prediction models using the compound covariate predictor, Bayesian compound covariate predictor, linear discriminator analysis, nearest centroid
classification, and support vector machines (18–20). The models incorporated genes that were differentially expressed between the two classes using a two-sample \( t \) test. Genes were considered to have statistically significant differences in expression if \( P < 0.001 \). We estimated the prediction error of each model using leave-one-out cross-validation (LOOCV); for each LOOCV training set, the entire model-building procedure was repeated, including the gene selection process. The validation procedure was performed using BRB-ArrayTools (version 4.3.2).

### Gene set and gene network analysis

A gene set enrichment analysis was performed to identify the most significant gene sets associated with the disease process, molecular and cellular functions, and physiologic and development conditions. The significance of overrepresented gene sets was estimated by the Fisher exact test. To explore the relationships between the genes in the newly identified signature, we generated gene networks based on whether they had more interconnected genes than would be expected to occur by chance. The significance of each network was estimated using a scoring system in which the scores were determined by the number of differentially expressed genes within each of the networks and the strength of the associations among the network members. Gene set enrichment and gene network-based activation regulator analyses were performed using the Ingenuity Pathway Analysis (IPA) tool.

### Other statistical analyses

The prognostic association between the signature and other known clinical and pathologic risk factors was assessed by multivariate Cox proportional hazard regression models. To estimate the significance of gene expression differences between the patient subgroups, we performed a two-sample \( t \) test for each gene. The statistical analysis was primarily performed using the R language environment (version 2.15.1).

### Cell culture

Human bladder cancer cell lines (EJ and 5637; refs. 21, 22) were obtained from the American Type Culture Collection.
Collection (ATCC). Other cell lines (UC5 and UC9; ref. 23) were provided by H. Barton Grossman (Department of Urology, University of Texas MD Anderson Cancer Center: deposit into Public Health England, United Kingdom). The cells in this study were used within 6 months in our laboratory, obtained from a cell bank that performed cell line characterizations. We were informed about the authentications of whole cell lines when obtained by the described providers: EJ and 5637 were certificated by the results of the tests [short tandem repeat (STR) DNA profiling assay, cytochrome C oxidase I assay, and Mycoplasma contamination]. UC5 and UC9 were characterized by STR-PCR method and Mycoplasma contamination.

UC5, UC9, and EJ cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; HyClone Laboratories, Inc.), 5637 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone Laboratories, Inc.), supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc.) and 1% penicillin–streptomycin (100 U/mL) at 37°C in a humid environment containing 5% CO2. Doxorubicin (Sigma-Aldrich) was dissolved in sterile water.

In our previous report, 5637 and EJ cells showed more invasive activity than UC5 and UC9 cancer cells by an invasion assay using Boyden chambers (24). Thus, to further investigate the association between gene expression signatures and bladder tumor aggressiveness, we selected two NMIBC-like (i.e., UC5 and UC9) and two MIBC-like (i.e., 5637 and EJ) bladder cancer cells and estimated the expression of forkhead box M1 (FOXM1) and cyclin B1 (CCNB1) mRNA in the cells.

Transfection and plasmid DNA

To determine whether FOXM1 regulates DNA repair genes, 5637 and UC5 cells were transiently transfected with FOXM1 expression vectors (constructed using forward, AT-GCAAGCTTGGTGAAGAG-GAAGCCCAT, and reverse, ATGCCCTGAGACCTTCCCA-CAGCCCTF primers) with the jetPRIME reagent (Polyplus-transfection Inc.); the ratio of DNA to jetPRIME was 1:3.

Real-time reverse transcriptase polymerase chain reaction analysis

To determine the effect of FOXM1 and doxorubicin on the endogenous expression levels of DNA repair genes, RNA (2 μg) was isolated from the bladder cancer cell lines using TRI Reagent (Ambion). The isolated RNA samples were reverse-transcribed and amplified using qRT-PCR with the primer sets described in Supplementary Table S1. For qRT-PCR, 50 ng of cDNA was analyzed using SYBR Green dye (Bio-Rad) and the CFX96 Optics Module system (Bio-Rad) with the following standard amplification protocol: 5 minutes at 95°C, followed by 10 seconds at 95°C and 30 sec at 60°C.

MTT assay

An MTT assay was used to analyze the survival of UC5 and 5637 cells after treatment with doxorubicin. A fixed number of cells were seeded in 96-well culture plates, and the cells were replenished with FBS containing culture medium with or without doxorubicin (1–50 μg/mL). The doxorubicin-treated cells (4,000 cells per well) were plated in 96-well culture plates for 24 hours. Briefly, 20 μL of the MTT reagent (5 mg/mL) was added to each well for 2 hours at 37°C. After the addition of 100 μL dimethyl sulfoxide (DMSO), the absorbance of each well was measured using a Wallac Victor 1420 Multilabel Counter (EG&G Wallac) at a wavelength of 540 nm.

Results

Prognostic utility of CCNB1 and its associated genes

To identify possible gene expression signatures responsible for the recurrence of NMIBC, we generated in trans gene sets correlated with all the gene features in the Korean cohort (Pearson correlation test: \( P < 0.001, r < -0.4 \) or \( r > 0.4 \)). On the basis of a hierarchical clustering analysis of the expression patterns of in trans genes correlated with each gene feature, we then divided the NMIBC samples into two groups and estimated the prognostic value of each gene set for the NMIBC recurrence by a log-rank test. Supplementary Table S2 shows the genes and number of their correlated genes that are strongly associated with disease recurrence in NMIBC (cutoff at \( P < 0.01 \) for the log-rank test and number of correlated genes >1,000).

CCNB1 was one of the genes strongly associated with disease recurrence (Supplementary Table S2). Because CCNB1 is frequently upregulated and has been used as a prognostic indicator in many cancers (25–27), we further attempted to estimate the predictive value of CCNB1 for the recurrence of NMIBC. As shown in Supplementary Table S2, we identified 1,393 genes with a change in expression that correlated with CCNB1 expression. We performed a hierarchical clustering analysis and divided the NMIBC samples into two groups: a high CCNB1 cluster (HC) and low CCNB1 cluster (LC). The recurrence rate of the HC patients was significantly higher than that of the LC patients (log-rank test: \( P < 0.001 \); Fig. 1).

To evaluate the prognostic efficacy of the newly identified signature based on CCNB1 expression, we applied a multivariate Cox regression analysis to the signature and known clinical and pathologic prognostic factors for NMIBC (Table 2). This analysis revealed that the molecular signature of CCNB1-associated genes [HR, 2.930; 95% confidence interval (CI), 1.302–6.594; \( P = 0.009 \)] was a strong predictor of bladder cancer recurrence.

Validation of CCNB1 signature in an independent cohort

We next sought to validate our findings using gene expression data from an independent cohort of European patients with bladder cancer (7). Using the CCNB1-associated gene signature, we applied previously established data training and prediction methods to test the accuracy of our signature-based prediction of disease recurrence (Supplementary Fig. S1). We identified the genes with the greatest difference in expression level between the HC and LC subgroups in the Korean cohort (the training set). These genes were pooled to form a series of classifiers able to...
estimate the probability that a particular bladder cancer sample belonged to the HC or LC subgroup. The number of genes in the classifier set was optimized to minimize misclassification during LOOCV of the tumors in the training set. The performance of each prediction model is illustrated in Supplementary Table S3. When applied to the European cohort (the test set), all models produced consistent prediction patterns, and the Kaplan–Meier estimations in the test set revealed significant differences in the risk of recurrence between the patients in subgroups HC and LC (Supplementary Fig. S1B).

In the European cohort, the prognostic association between the signature and other known clinical and pathologic risk factors for recurrence-free survival in NMIBC was also evaluated by a multivariate Cox regression analysis (Supplementary Table S4). Similar to the Korean cohort, this analysis revealed that the signature remained an independent risk factor for recurrence-free survival (HR, 5.277; 95% CI, 2.479–11.235; P < 0.001). These results not only demonstrate a strong association between gene expression patterns and disease recurrence but also provide strong evidence of the reliability of the prediction.

The **CCNB1** signature is an independent risk factor for disease recurrence in NMIBC

To estimate the independence of the **CCNB1** signature over the current known prognostic variables, such as the stage or grade, gene expression data from the Korean and European cohorts were pooled (n = 455), and the patients were stratified according to these two modalities (stage and grade). When the signature-based stratification was applied to stage Ta and T1 separately, we successfully identified a population of high-risk patients in both stages (log-rank test: each P < 0.001, respectively; Fig. 2A). This finding strongly demonstrates that our new prognostic gene expression signature is independent of the current staging system. We also assessed the utility of the signature in patients with NMIBC who differed only in grade (low and high grade) and found that the frequency of recurrence in both grades was significantly higher for those in the HC patient group than those in the LC patient group (log-rank test: each P < 0.001, respectively; Fig. 2B). Taken together, these results demonstrate that the **CCNB1** signature is a powerful predictor of disease recurrence in patients with NMIBC, regardless of the current prognostic criteria.

**Significant association of the **CCNB1** signature with NMIBC recurrence after IVT**

Because adjuvant IVT with BCG and MMC constitutes the best treatment option for the prevention or delay...
of recurrence in high-risk NMIBC (2–4), we next sought to determine whether the CCNB1 signature and its associated molecular subgroups could predict a potential benefit from IVT. Thus, to assess the predictive value of the signature for pooled data from the Korean and European cohorts, we divided the patients with NMIBC into two subgroups (HC and LC) and independently estimated the difference in disease recurrence in each group. Importantly, we found that IVT significantly affected recurrence-free survival in the patients in subgroup HC ($P < 0.001$ by log-rank test), whereas we did not observe any significant association between the gene expression signature and IVT in the LC subgroup patients ($P = 0.254$ by log-rank test; Fig. 2C). Because treatment options (BCG, MMC, or both) of IVT were available from the European cohort, we further assessed the association between the CCNB1 signature and IVT categories. When applying substratifications, the patients in the HC subgroup had significant benefit for intravesical therapies including BCG, MMC, or both (log-rank test: $P < 0.001$; Supplementary Fig. S2A). However, we did not observe any significant association between the signature and IVT in the LC subgroup (log-rank test: $P = 0.063$; Supplementary Fig. S2B). Because most patients received BCG treatment and the cases with MMC or both treatments were very small, larger patient cohorts with more cases of MMC treatment are needed to determine clear sensitivity of MMC.

**Biologic characteristics of the prognostic and predictive CCNB1 signature**

To explore the biologic characteristics that are active in the NMIBC recurrence, a gene set enrichment test of the 1,393 genes featured in the recurrence signature (Fig. 1) was performed using IPA software. When applying the 1,393 genes to IPA, genes involved in cancer, cell cycle, and tissue development were found to be significantly enriched. Among the genes associated with disease recurrence in NMIBC, a significant number was identified as involved in DNA replication, recombination, and repair, indicating that the biologic processes associated with the DNA repair system might markedly affect the heterogeneous clinical behavior of NMIBC (Supplementary Fig. S3).

To identify the predominant regulators and signaling pathways active in NMIBC recurrence, upstream regulator analyses of the 1,393 genes were also performed using IPA. An examination of the enriched genes revealed the involvement of several important activated regulators (Supplementary Table S5), the strongest overrepresentation of which was the predominant activation of FOXM1 (Supplementary Fig. S4). FOXM1 is an oncogenic transcription regulator of which CCNB1 is a downstream effector. FOXM1 formed the primary hub of the gene network that was subsequently interconnected with another gene network hub composed by CCNB1. All of the satellite genes connected to FOXM1 or CCNB1 (Supplementary Fig. S4) participate in DNA replication, recombination, and repair, corresponding to the best-known activities of FOXM1 and CCNB1. The expression levels of CCNB1 and FOXM1 were significantly higher in the HC versus the LC subgroup of the Korean cohort (two-sample $t$ test: each $P < 0.001$, respectively; Supplementary Fig. S5A). We also examined the expression levels of CCNB1 and FOXM1 in the two independent patient cohorts (the SSH ($n = 213$) and SUH ($n = 92$) cohorts). The patients in these cohorts were divided into two groups (HC and LC patient groups) by hierarchical cluster analyses using the 1,393 genes derived from the Korean cohort (Supplementary Fig. S5B). Similar to the Korean cohort, the expression levels of CCNB1 and FOXM1 in the HC subgroup were significantly higher than those in the LC subgroup in the two independent cohorts (two-sample $t$ test: each $P < 0.001$, Supplementary Fig. S5C). These results indicate that the activation of the
FOXM1–CCNB1 signaling network may be a key genetic determinant associated with a poor prognosis of NMIBC subgroup HC patients. Interestingly, among the downstream genes affected by CCNB1, a number of genes from the Fanconi anemia (FA) family (i.e., FANCA, FANCC, and FANCD2) were indirectly connected to CCNB1 (Supplementary Fig. S4). The expression of these FA family genes was significantly higher in the HC subgroup in the Korean cohort (two-sample t test: \( P < 0.001 \); Supplementary Fig. S6A), and we also found that FA family genes were more highly expressed in the HC groups in the SSH and SUH cohorts (two-sample t test: each \( P < 0.001 \); Supplementary Figs. S6B and S6C). These results indicate that the activation of FA pathway might, in part, account for the poorer prognosis of NMIBC subgroup HC.

**Experimental confirmation of the association between the CCNB1 signature and tumor aggressiveness**

We then performed a number of in vitro assays to verify whether the CCNB1 signature reflects the aggressive characteristics of bladder cancer. We first examined the gene expression levels of FOXM1 and CCNB1 and compared them between primary and recurrent NMIBCs. When comparing the microarray gene expression data, the expression levels of FOXM1 and CCNB1 in the recurrent tumor group were significantly higher than the primary tumor group (\( P = 0.004 \) and \( P = 0.002 \) by a two-sample t test, respectively, Fig. 3A). We also performed another gene expression analysis using RT-PCR and found significant expression differences for FOXM1 and CCNB1 between the primary and recurrent tumor groups.

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**Figure 3.** Comparison of expression levels of FOXM1 and CCNB1 in the tumor groups. A, two group box plots comparing expression levels of FOXM1 and CCNB1 in the microarray data. B, two group box plots comparing expression levels of FOXM1 and CCNB1 in the RT-PCR analysis. P values were obtained by two-sample t test between primary and recurrent tumor groups. Y-axis indicates median-centered gene expression of each gene (log2-transformed scale). C, expression levels of FOXM1 and CCNB1 in the bladder cancer cells (UC5, UC9, 5637, and EJ). ‘*’ > 2 fold.
NMIBCs, consistent with the microarray data ($P = 0.001$ and $P = 0.008$ by a two-sample t test, respectively, Fig. 3B), suggesting that FOXM1 and CCNB1 might account for relapse in NMIBC. To further investigate the association between the gene expression signature and bladder tumor aggressiveness, we selected two NMIBC-like (i.e., UC5 and UC9) and two MIBC-like (i.e., 5637 and EJ) bladder cancer cell lines and estimated FOXM1 and CCNB1 mRNA expression in these cells. FOXM1 and CCNB1 were more highly expressed in the 5637 and EJ cells than in the UC5 and UC9 cells (Fig. 3C), indicating that FOXM1 and CCNB1 might well reflect the aggressiveness of bladder cancer.

As FOXM1 and CCNB1 directly regulate DNA repair genes (Supplementary Fig. S4) and FOXM1 is involved in drug resistance by enhancing DNA repair (24), we also examined whether DNA repair genes, including FOXM1 and CCNB1, were associated with the aggressiveness of bladder cancer cells in drug-treated environments. We performed cell viability assays using UC5 and 5637 cells after doxorubicin treatments at 12 and 24 hours. As expected, increased the duration of doxorubicin treatment remarkably reduced the viability of the bladder cancer cells (Fig. 4A); however, the expression levels of FOXM1 and CCNB1 were significantly increased in these cells with the increased treatment duration (Fig. 4B). Interestingly, when estimating the expression of DNA repair genes with increasing doxorubicin treatment, the expression levels of FANCB and FANCD2, of the FA family, were highly increased compared with other DNA repair genes (Fig. 4C). These results demonstrate that the activity of FOXM1, CCNB1, and DNA repair genes involved in the FA pathway may be responsible for tumor aggressiveness, even with drug treatment. Finally, to explore the relationships among DNA repair genes, we performed overexpression assays of FOXM1 and CCNB1 and estimated the changes in the expression of other DNA repair genes in the 5637 cells. Importantly, when FOXM1 was overexpressed, the genes in the FA pathway (i.e., FANCB, FANCC, and FANCD2) were highly expressed compared with the control (Fig. 4D). In contrast, other DNA repair genes not involved in the FA pathway did not show significant changes in expression. We obtained similar significant results with CCNB1 overexpression (Fig. 4D). These results suggest that the aggressive characteristics of bladder cancer may be mediated by the FA pathway, as regulated by the activation of the FOXM1–CCNB1 signaling network.

Discussion

The recurrence of NMIBC may be affected by several complicated molecular interactions, whereby certain genes may mainly drive disease events and other genes may have close cooperation with them. On the basis of an iterative in trans correlation approach, we showed that the expression signature defined by CCNB1 and its associated genes was able to predict the likelihood of recurrence in NMIBC. The validity of this signature as a prognostic indicator was confirmed by the analysis of other NMIBCs from independent patient cohorts. We also showed the prognostic value of CCNB1-correlated genes as an independent prognostic signature compared with other clinicopathologic factors and its predictive value for IVT. In addition, based on the results of our gene network analysis, putative signaling pathways that might be responsible for NMIBC recurrence were identified, and their association with tumor aggressiveness was confirmed by a number of in vitro assays (Supplementary Fig. S7).

There is an evident clinical heterogeneity among patients with NMIBC. Most patients with NMIBC receive standard treatments of TUIR, followed by adjuvant intravesical chemotherapy or immunotherapy with BCG or MMC to prevent disease relapse. However, a significant number of patients with NMIBC fail to respond to IVT and frequently experience disease recurrence (3–5). Although considerable efforts have been devoted to the establishment of a prognostic model of NMIBC that can provide information about survival and treatment options at diagnosis, the ability to predict the clinical course of disease recurrence and response to IVT for NMIBC remains a major clinical challenge. In the present study, we developed a method to predict the recurrence of primary NMIBC based on a gene expression signature that consisted of CCNB1 and its associated genes (Fig. 1). On the basis of the CCNB1 signature, the patients with NMIBC classified in the HC subgroup had a potential benefit from IVT, whereas those in the LC subgroup did not (Fig. 2C). These data underscore the importance of the HC molecular subgroup defined by the CCNB1 signature as a potential prognostic and predictive subtype in NMIBC.

Several lines of evidence strongly support the CCNB1 signature as an independent and significant predictor of recurrence in NMIBC. First, a subset analysis of patients by tumor stage showed that the signature was able to identify high-risk patients at both stages Ta and T1 (Fig. 2A). Second, through another subset analysis of patients by tumor grade, the CCNB1 signature was found to be an independent predictor of disease recurrence in patients with NMIBC, regardless of the current grading system (Fig. 2B). Third, the CCNB1 signature was a significant predictive factor for recurrence in both the exploration and validation cohorts according to multivariate analyses (Table 2 and Supplementary Table S4). Taken together, these results suggest that the CCNB1 signature strongly retains its prognostic relevance, even after additional pathologic prognostic features have been taken into account.

On the basis of our analysis of the CCNB1 signature within the context of gene networks, we identified putative signaling pathways significantly associated with disease recurrence in bladder cancer. The network analysis revealed that many DNA repair genes, including CCNB1 under the control of FOXM1 activity, were significantly activated in NMIBC recurrence (Supplementary Fig. S4). FOXM1 is frequently associated with metastasis and patient survival in many types of cancers (28, 29); it was also reported that FOXM1 activity was significantly increased in bladder cancers relative to normal bladder tissues and might have a prognostic value at the individual level (30). CCNB1, a
downstream effector of FOXM1, is upregulated and is known to be a prognostic biomarker in many cancers (25–27). Interestingly, several genes involved in the FA pathway were regulated by CCNB1 (Supplementary Fig. S4), and their activities were experimentally confirmed using overexpression assays in doxorubicin-induced bladder cancer cells (Fig. 4). These genes are known to be involved in the cell-cycle checkpoint and DNA repair that the FA/BRCA pathway regulates by homologous recombination (31). FA is a genetic disorder that arises from defects in the proteins responsible for DNA repair, and a vast majority of FA patients develop aggressive cancer, such as acute myeloid leukemia with bone marrow failure (32). Taken together, we suggest that putative signaling via FOXM1, CCNB1, and the FA pathway activated in subgroup HC bladder cancer patients may well reflect the

Figure 4. Expression changes in DNA repair genes after doxorubicin treatment of bladder cancer cells. A, viability of the UC5 and 5637 cells after doxorubicin treatment at 12 and 24 hours. B, expression changes in FOXM1 and CCNB1 with increasing doxorubicin treatment in the UC5 and 5637 cells. C, expression changes in DNA repair genes with increasing doxorubicin treatment in the UC5 and 5637 cells. D, expression changes in DNA repair genes when FOXM1 and CCNB1 were overexpressed in the 5637 cells. *, >1.2 fold; **, >1.5 fold; ***, >2 fold.
aggressiveness of NMIBC. The use of the CCNB1 signature identified in the present study as a predictive indicator could potentially enable the more accurate prognosis of patients with heterogeneous NMIBC at diagnosis, which would allow for individualized treatment and evaluation.

In conclusion, based on an expression signature comprised of FOXM1, CCNB1, and its associated genes, we identified two new prognostic subgroups of NMIBC that show a significant difference in NMIBC recurrence. Our results also demonstrate that the signature can predict the response to IVT.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.-K. Kim, S.-H. Leem, I.-S. Chu
Development of methodology: S.-K. Kim, I.-S. Chu

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References


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