Expression signature defined by \textit{FOXM1-CCNB1} activation predicts disease recurrence in non-muscle-invasive bladder cancer

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
**Translational Relevance**

Non-muscle-invasive bladder cancer (NMIBC) is clinically heterogeneous, and many patients with NMIBC fail to respond to treatment and frequently experience disease recurrence. In this study, an expression signature activated by FOXM1-CCNB1 was identified for predicting NMIBC recurrence and response to intravesical therapy. The signature to discriminate distinct molecular subgroups of NMIBC was developed in a training cohort and was validated in independent cohorts. The signature was recurrence-free survival predictor independent, with traditional clinico-pathological factors. The patients in a subgroup classified by the signature exhibited a potential benefit of intravesical immunotherapy, providing prognostic and predictive value of the newly identified signature. Through experimental assays, we also found that NMIBC recurrence could be mediated by FOXM1-CCNB1-Fanconi anemia pathways. Identification of a high-risk subgroup of patients with NMIBC based on the signature mediated by FOXM1-CCNB1 might improve the current molecular classification system of bladder cancer.
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 NMIBC patients 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 NMIBC patients 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% CI = 1.302 to 6.594, P = 0.009). The subset analysis also revealed that the signature could identify patients who would benefit from IVT. Lastly, 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 NMIBC patients who have a high risk of recurrence and to predict response to IVT.
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 over 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 cancer patients 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 biological 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 NMIBC patients 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 NMIBC patients from hospitals of the Swedish southern healthcare region (GSE32894, the SSH cohort, n = 213) (16) and Skåne University Hospital (GSE32549, the SUH cohort, n = 92) (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 \textit{in trans} correlation analysis

To generate an \textit{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 genes having significant correlation coefficients with an initial gene feature across NMIBC patients ($|r| > 0.4$ 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 exist in the gene expression data from the Korean cohort. A $P$-value of $<0.01$ 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 the $P$-value $< 0.001$. 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 physiological and development conditions. The significance of over-represented gene sets was estimated by Fisher’s 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 Ingenuity Pathway Analysis™ (IPA) Tool.

**Other statistical analyses**

The prognostic association between the signature and other known clinical and pathological 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) (21, 22) were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Other cells lines (UC5 and UC9) (23) were provided by H. Barton Grossman (Department of Urology, University of Texas M. D. Anderson Cancer Center; deposit into Public Health England, UK). The cells in this study
were used within 6 months in our lab, obtained from a cell bank that performed cell line characterizations. We were informed about the authentications of whole cell lines when obtained them 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 DMEM medium (Hyclone, Logan, UT), 5637 cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin-streptomycin (100 unit/ml) at 37°C in a humid environment containing 5% CO2. Doxorubicin (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water.

In previous our 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 (24) and *CCNB1* expression vectors (constructed using F, AT-GCAAGCTTGGTGAAGAGGAAGCCATG, and R, ATGCCTCGAGACCTTTGCCACAGCCATG, primers) with the jetPrime reagent (Polyplus-transfection Inc., New York, NY); the ratio of DNA to jetPrime was 1:3.

**Real-time reverse transcriptase polymerase chain reaction (RT-PCR) 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, Carlsbad, CA). The isolated RNA samples were reverse-transcribed and amplified using quantitative real-time PCR with the primer sets described in Supplementary Table S1. For quantitative real-time PCR, 50 ng of cDNA was analyzed using SYBR Green dye (Bio-Rad, Hercules, CA) and the CFX96TM Optics Module system (Bio-Rad Inc., Foster City, CA) with the following standard amplification protocol: 5 min at 95°C, followed by 10 sec 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 (4000 cells/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 DMSO, the absorbance of each well was measured using a Wallac Victor 1420 Multilabel Counter (EG&G Wallac, MD) 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 $r > 0.4$). Based on 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 (cut-off 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 up-regulated 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 (hazard ratio 2.930, 95% confidence
interval = 1.302 to 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 pathological 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 (hazard ratio 5.277, 95% confidence interval = 2.479 to 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 NMIBC patients, 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 NMIBC patients 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 \( (P = 0.254 \text{ by log-rank test}) \) (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 sub-stratifications, 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.

**Biological characteristics of the prognostic and predictive \( CCNB1 \) signature**

To explore the biological 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 biological 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 over-representation 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 \textit{CCNB1}. All of the satellite genes connected to \textit{FOXM1} or \textit{CCNB1}
(Supplementary Fig. S4) participate in DNA replication, recombination, and repair,
corresponding to the best-known activities of \textit{FOXM1} and \textit{CCNB1}. The expression levels of
\textit{CCNB1} and \textit{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 \textit{CCNB1} and \textit{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 \textit{CCNB1} and \textit{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 \textit{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 \textit{CCNB1}, a number of genes from the Fanconi anemia (FA)
family (i.e., \textit{FANCB}, \textit{FANCC}, and \textit{FANCD2}) were indirectly connected to \textit{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.

\textbf{Experimental confirmation of the association between the \textit{CCNB1} signature and tumor}

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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 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, increasing 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 to 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. Lastly, to explore the relationships among DNA repair genes, we performed over-expression 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 over-expressed, the genes in the FA pathway (i.e., FANCB, FANCC, and FANCD2) were highly expressed compared to 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 over-expression (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. Based on 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 to other clinico-pathological 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 NMIBC patients. Most patients with NMIBC receive standard treatments of TUR, 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 concerning 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). Based on the CCNB1 signature, the NMIBC patients 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 \textit{CCNB1} signature as a potential prognostic and predictive subtype in NMIBC.

Several lines of evidence strongly support the \textit{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 \textit{CCNB1} signature was found to be an independent predictor of disease recurrence in NMIBC patients, regardless of the current grading system (Fig. 2B). Third, the \textit{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 \textit{CCNB1} signature strongly retains its prognostic relevance, even after additional pathological prognostic features have been taken into account.

Based on our analysis of the \textit{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 \textit{CCNB1} under the control of \textit{FOXM1} activity, were significantly activated in NMIBC recurrence (Supplementary Fig. S4). \textit{FOXM1} is frequently associated with metastasis and patient survival in many types of cancers (28, 29); it was also reported that \textit{FOXM1} activity was significantly increased in bladder cancers relative to normal bladder tissues and might have a prognostic value at the individual level (30). \textit{CCNB1}, a downstream effector of \textit{FOXM1}, is up-regulated and is known to be a prognostic biomarker in many cancers (25-27). Interestingly, several genes involved in the FA pathway were regulated by \textit{CCNB1} (Supplementary Fig. S4), and their activities were experimentally confirmed using over-expression assays in doxorubicin-induced bladder cancer cells (Fig. 4). These genes are
known to be involved in the cell-cycle checkpoint and DNA repair which the FA/BRC 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 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 heterogeneous NMIBC patients 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.
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References


# Tables

## Table 1. Baseline characteristics of NMIBC patient cohorts.

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<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>66</td>
<td>69</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>Range</td>
<td>24-88</td>
<td>27-95</td>
<td>20-93</td>
<td>38-89</td>
</tr>
<tr>
<td>Stage, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>23 (22.5)</td>
<td>189 (53.5)</td>
<td>116 (54.5)</td>
<td>40 (43.5)</td>
</tr>
<tr>
<td>T1</td>
<td>79 (77.5)</td>
<td>164 (46.5)</td>
<td>97 (45.5)</td>
<td>52 (56.5)</td>
</tr>
<tr>
<td>Grade, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>85 (83.3)</td>
<td>97 (27.5)</td>
<td>139 (65.3)</td>
<td>52 (56.5)</td>
</tr>
<tr>
<td>High</td>
<td>17 (16.7)</td>
<td>223 (63.2)</td>
<td>72 (33.8)</td>
<td>40 (43.5)</td>
</tr>
<tr>
<td>NA</td>
<td>33 (9.3)</td>
<td>2 (0.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0 (0)</td>
<td>51 (14.4)</td>
<td>10 (10.9)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>102 (100)</td>
<td>302 (85.6)</td>
<td>77 (83.7)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td></td>
<td>213</td>
<td>5 (5.4)</td>
<td></td>
</tr>
<tr>
<td>Intravesical Therapy, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>46 (45.1)</td>
<td>90 (25.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>56 (54.9)</td>
<td>263 (74.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td></td>
<td>213</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Recurrence, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36 (35.3)</td>
<td>91 (25.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>66 (64.7)</td>
<td>262 (74.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td></td>
<td>213</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Median RFS (months)</td>
<td>30.8</td>
<td>42.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>55.3</td>
<td>52.0</td>
<td>37.9</td>
<td>58.0</td>
</tr>
</tbody>
</table>

NMIBC, non-muscle-invasive bladder cancer; NA, not available; RFS, recurrence-free survival; SSH, Swedish southern healthcare region; SUH, Skåne University Hospital; CIS, carcinoma in situ.
### Table 2. Multivariate Cox regression analysis for the prediction of disease recurrence in the Korean cohort.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Recurrence</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>Gender (Male vs. Female)</td>
<td>0.983 (0.398 - 2.432)</td>
<td>0.971</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.995 (0.968 - 1.022)</td>
<td>0.695</td>
<td></td>
</tr>
<tr>
<td>Stage (Ta vs. T1)</td>
<td>1.178 (0.568 - 2.442)</td>
<td>0.660</td>
<td></td>
</tr>
<tr>
<td>Grade (Low vs. High)</td>
<td>1.012 (0.562 - 1.824)</td>
<td>0.967</td>
<td></td>
</tr>
<tr>
<td>Tumor size (≤ 3 cm vs. &gt; 3 cm)</td>
<td>1.006 (0.363 - 2.792)</td>
<td>0.990</td>
<td></td>
</tr>
<tr>
<td>Number of tumors (single vs. multiple)</td>
<td>0.871 (0.339 - 2.242)</td>
<td>0.775</td>
<td></td>
</tr>
<tr>
<td>Intravesical therapy (No vs. Yes)</td>
<td>0.331 (0.135 - 0.809)</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>CCNB1 signature (LC vs. HC*)</td>
<td>2.930 (1.302 - 6.594)</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

*Outcome from the unsupervised hierarchical clustering in Fig. 1 was used for the analysis (low CCNB1 cluster [LC] or high CCNB1 cluster [HC]).

Abbreviations: HR, hazard ratio; CI, confidence interval.
Figure Legends

Figure 1. Gene expression pattern of the CCNB1 signature and recurrence-free survival of two clusters in the Korean cohort (n = 102). A, the gene expression patterns of CCNB1 and its associated genes. A total of 1,393 genes with expression patterns that highly correlate with CCNB1 were selected for a cluster analysis (Pearson correlation test, \( P < 0.001, r < -0.4 \) or \( r > 0.4 \)). The patients were divided into two groups: a high CCNB1 cluster (HC) and low CCNB1 cluster (LC). B, Kaplan-Meier curves showing time to recurrence. The recurrence rate of the HC patients was significantly higher than that of the LC patients (log-rank test, \( P < 0.001 \)).

Figure 2. Kaplan-Meier plots of disease recurrence in the patients in the combined cohort. A, a subset analysis by stage. The subgroups based on the CCNB1 signature were predictive in the stage Ta and T1 patients. B, a subset analysis by grade. The subgroups based on the CCNB1 signature were predictive in the low- and high-grade patients. C, prediction of a response to intravesical therapy in the two subgroups with the CCNB1 signature. Patients in the HC subgroup had significant benefit from intravesical therapy. The data were plotted according to whether the patients received intravesical therapy. LC, low CCNB1 cluster; HC, high CCNB1 cluster; LG, low-grade; HG, high-grade; IVT, intravesical therapy.

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.

**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 over-expressed in the 5637 cells. *, > 1.2 fold; **, > 1.5 fold; ***, > 2 fold.
Figure 1

(A) Heatmap of CCNB1 expression in different clusters. Red indicates high CCNB1 expression, while green indicates low expression.

(B) Recurrence-free survival curve comparing high (HC) and low (LC) CCNB1 clusters. The Kaplan-Meier curve shows a significant difference between the two groups (P < 0.001).

LC(n=50, 10R)

HC(n=52, 26R)
Expression signature defined by FOXM1-CCNB1 activation predicts disease recurrence in non-muscle-invasive bladder cancer

Seon-Kyu Kim, Yun-Gil Roh, Kiejung Park, et al.

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