The 19q12 Bladder Cancer GWAS Signal: Association with Cyclin E Function and Aggressive Disease

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The 19q12 Bladder Cancer GWAS Signal: Association with Cyclin E Function and Aggressive Disease

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Abstract

A genome-wide association study (GWAS) of bladder cancer identified a genetic marker rs8102137 within the 19q12 region as a novel susceptibility variant. This marker is located upstream of the CCNE1 gene, which encodes cyclin E, a cell-cycle protein. We performed genetic fine-mapping analysis of the CCNE1 region using data from two bladder cancer GWAS (5,942 cases and 10,857 controls). We found that the original GWAS marker rs8102137 represents a group of 47 linked SNPs (with $r^2 > 0.7$) associated with increased bladder cancer risk. From this group, we selected a functional promoter variant rs7257330, which showed strong allele-specific binding of nuclear proteins in several cell lines. In both GWASs, rs7257330 was associated with aggressive bladder cancer, with a combined per-allele OR = 1.18 [95% confidence interval (CI), 1.09–1.27, $P = 4.67 \times 10^{-5}$] versus OR = 1.01 (95% CI, 0.93–1.10, $P = 0.79$) for nonaggressive disease, with $P = 0.0015$ for case-only analysis. Cyclin E protein expression analyzed in 265 bladder tumors was increased in aggressive tumors ($P = 0.013$) and, independently, with each rs7257330-A risk allele ($P_{\text{trend}} = 0.024$). Overexpression of recombinant cyclin E in cell lines caused significant acceleration of cell cycle. In conclusion, we defined the 19q12 signal as the first GWAS signal specific for aggressive bladder cancer. Molecular mechanisms of this genetic association may be related to cyclin E overexpression and alteration of cell cycle in carriers of CCNE1 risk variants. In combination with established bladder cancer risk factors and other somatic and germline genetic markers, the CCNE1 variants could be useful for inclusion into bladder cancer risk prediction models.

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Introduction

Bladder cancer is the sixth most common cancer in the United States, with an estimated 72,570 new cases and 15,210 deaths in 2013 (1). At presentation, bladder cancer is classified into non–muscle-invasive (NMIBC), muscle-invasive (MIBC), or metastatic disease. Most of bladder cancer cases present as NMIBC (70%), which are low or high-grade tumors with stages Ta, T1, or carcinoma in situ (TIS). Low-grade NMIBC (tumor stage Ta with grade G1/G2) has a high rate of recurrence but a low rate of progression to MIBC, whereas high-grade NMIBC (stage Ta with grade G3/G4, all stage T1 and TIS) has a high rate of recurrence and progression to MIBC (stages T2–T4 with any grade). Patients with NMIBC are treated by bladder-sparing therapies and require frequent expensive surveillance but the disease is not life threatening. MIBC cases are primarily treated with surgical resection of the bladder (radical cystectomy), but 50% of patients still progress to incurable metastatic disease (2). Bladder cancer is considered a clinically heterogeneous disease, with low-grade NMIBC classified as nonaggressive and genomics stable, whereas high-grade NMIBC and MIBC classified as aggressive and genomics unstable (3, 4).

It is anticipated that some of the genetic markers discovered by genome-wide association studies (GWAS) will contribute to improved understanding of relationships between inherited susceptibility and molecular mechanisms of cancer and may become clinically useful for risk prediction models. Our recent study of bladder cancer (NCI-GWAS; ref. 5), performed in individuals of European ancestry, identified several novel susceptibility loci, including an SNP rs8102137 within the 19q12 region. Of all bladder cancer GWAS signals reported to date (5–8), only rs8102137 has been specifically associated with risk of high-grade but not low-grade tumors (5).

The GWAS marker rs8102137 is located 6 Kb upstream of the CCNE1 gene, which encodes cyclin E, a cell-cycle protein. As the only gene located within the associated linkage disequilibrium (LD) block, CCNE1 is a primary functional candidate gene for this GWAS signal. The cyclin E protein forms a complex with cyclin-dependent kinase 2 (CDK2) and regulates the transition of cell cycle and further progression through S phase (9). Increased cyclin E expression is found in many tumor types, including breast, gastric, colorectal, ovarian, and bladder cancers (10). Cyclin E is a short-lived unstable protein quickly destroyed by proteolytic degradation (11). Therefore, its high protein expression must be sustained by increased mRNA expression, which can be affected by a number of factors, including somatic mutations, genomic amplifications (12), or germline genetic variants (13) within the CCNE1 region. However, somatic mutations of the CCNE1 gene were found only in 44 (0.49%) of 8,904 tumors of different types in the COSMIC database (14) and thus are unlikely to have significant effects on cyclin E function.

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Here, we searched for germline genetic variants that could explain the initial GWAS signal within the 19q12 region and explored association of these variants with informative molecular phenotypes, such as \(CCNE1\) mRNA and protein expression in bladder tissues.

Materials and Methods

Fine-mapping analysis

We used genotyping data from two bladder cancer GWASs conducted by the U.S. NCI. NCI-GWAS1 included 3,520 bladder cancer cases and 5,110 controls (5), and NCI-GWAS2 included 2,422 cases and 5,747 controls (6; Supplementary Table S1). The use of GWAS data was approved by ethic committees of corresponding studies (5, 6). Imputation-based fine mapping of the approximately 70-Kb \(CCNE1\) region (±30 Kb around the \(CCNE1\) gene, genomic coordinates GRCh37, chr19: 30,272,901-30,345,215) was performed using data from both NCI-GWASs as previously described (15), based on the 1000 Genomes Project data (phase 1 version 3, 2012 March revised; ref. 16) using IMPUTE2 (17). We analyzed only well-imputed variants (IMPUTE2 info score ≥0.9) and exonic nonsynonymous variants regardless of imputation score. Imputation results for rs7257330 were validated by TaqMan genotyping (assay C_32389893_20; Life Technologies) in 336 NCI-GWAS1 samples (99.4% concordance). Imputed genotypes of rs61750863 were tested by Sanger sequencing in 608 samples (Supplementary Table S3 for primers and Supplementary Table S7 for results).

Cell lines and tissue samples

Cell lines HeLa (cervical carcinoma) and HTB5 (bladder transitional carcinoma) were from the ATCC. Cell lines were last authenticated in July 2014 by the DNA Diagnostic Center (DDC Medical, Fairfield, OH) based on genomic analysis of a panel of short tandem repeats (STR) and comparison with the ATCC STR Profile Database. Fresh-frozen bladder tissues (41 tumors and 40 adjacent normal samples) and 17 formalin-fixed paraffin-embedded tumors (Supplementary Table S2) were purchased from Asterand (Detroit) after exemption #4715 provided by the NIH Office of Human Subjects Research (15).

mRNA expression and IHC protein analyses

RNA sequencing of bladder tumors and adjacent normal bladder tissue samples (Supplementary Materials) has been described (15). Quantitative reverse-transcriptase PCR (qRT-PCR) analysis of mRNA expression in fresh-frozen tissue samples was performed with TaqMan assays as described (Supplementary Table S3 and Supplementary Materials). IHC analysis of bladder tissue microarrays (TMA) was performed as described (18, 19). A pilot custom TMA included 8 normal-tumor bladder tissue pairs and additionally, 1 unpaired bladder tumor, and 3 prostate tumors (Asterand). Another TMA set of 265 samples included bladder tumors from patients from the New England Bladder Cancer Study, NEBCS (18, 19), with available GWAS data, stage, and grade information and sufficient quantity and quality of tumor tissue. IHC was performed using standard polymer-based immunohistochemical methods using antibodies and conditions presented in Supplementary Table S3. Cyclin E staining was scored manually by a pathologist (P. Lenz) blinded to clinical and genotype information. Cyclin E nuclear staining was assessed as percentage of positive cells (0: negative, 1: <10%, 2: 10%–50%, 3: >50% of cells) and nuclear staining intensity (0: negative, 1: weak, 2: moderate, 3: strong). A combined cyclin E nuclear staining score ranging from 0 to 6 was a sum of the scores for nuclear positivity and intensity (Fig. 5B). The cyclin E cytoplasmic expression was scored based on intensity only (0: negative, 1: weak, 2: moderate, 3: strong).

Statistical analysis

Association between imputed markers and bladder cancer risk was evaluated with SNPTEST v2 based on estimated allelic dosage from IMPUTE2. Per-allele ORs and \(P\) values were calculated adjusting for age, gender, smoking status (ever/never), study sites, and main eigenvectors (EV1, EV5, and EV6) used in NCI-GWAS datasets to adjust for possible population stratification among the sub-studies. Residual association signals in the \(CCNE1\) region were explored by conditioning on the original GWAS marker rs8102137 in the logistic regression models. Best-guessed genotypes of imputed markers with ≥0.9 probability were converted by GTOOL and used for calculation of LD values (\(D^2\) and \(r^2\)) and for detailed stratified analyses. For final analysis, bladder cancer aggressiveness was defined into two categories: the nonaggressive category included cases with low-grade NMIBC (Ta with G1/G2), and the aggressive category included cases with high-grade NMIBC (Ta with G3/G4 or any T1) and MIBC (all T2−T4; Supplementary Table S4). Differences in effect sizes between the bladder cancer categories were evaluated in case-only analysis.

Differences in mRNA expression levels between bladder tumors and adjacent normal tissue samples were evaluated with the unpaired Student \(t\) test. The association between \(CCNE1\) mRNA expression and SNP genotypes was evaluated with multivariable linear regression models assuming additive genetic effects and adjusting for age and gender. The association between genotypes and IHC scores (0−6 scale) was evaluated by multivariable linear models assuming additive genetic effects, with adjustment for age, gender, study centers, smoking, and bladder cancer aggressiveness. Meta-analysis and forest plots were generated with STATA version 11 (StatCorp LP). All statistical tests were two-sided and conducted with SAS/STAT system version 9.2 (SAS Institute Inc.) unless otherwise specified. Graphs were plotted with Prism 6 software (GraphPad Inc.). All the tests were two-sided.

Functional assays

Electrophoretic mobility shift assays, cloning of \(CCNE1\) transcripts, cell cycle, and protein interaction analyses are described in Supplementary Table S3 and Supplementary Materials.

Results

Genetic association of \(CCNE1\) region with aggressive bladder cancer

Imputation in the combined GWAS set generated a list of 152 well-imputed markers in addition to the 13 GWAS-genotyped SNPs located within the \(CCNE1\) region. Of these 165
SNPs, 46 markers were in strong ($r^2 \geq 0.7$) LD with rs8102137 and comparably represented the original GWAS signal (Supplementary Table S5).

Within the group of 47 linked CCNE1 markers, we focused on a promoter SNP rs7257330, which has high correlation with rs8102137 ($r^2 = 0.77$). This variant resides 1.1 Kb upstream of the CCNE1 transcription start site, within a genomic area with a uniquely strong histone H3 acetylation mark at Lysine 27 (H3K27Ac), a characteristic of active enhancers (20; Fig. 1). Experimental analysis of DNA–protein interactions using nuclear extracts from several human cell lines—HeLa (cervical carcinoma), J82 (bladder transitional carcinoma), and LNCaP (prostate cancer)—showed strong binding of the probe with risk rs7257330-A allele in all cell lines, whereas no binding was detected for the probe with nonrisk rs7257330-G allele (Supplementary Fig. S1A). Only a weak nonspecific interaction was detected for both alleles of the GWAS rs8102137 (Supplementary Fig. S1B). Bioinformatic analysis predicted that rs7257330 alleles may bind a number of proteins (Supplementary Fig. S1C), precluding experimental testing of all these predictions.

For analysis of genetic association between CCNE1 variants and bladder cancer subtypes, we used a classification based on the combined tumor stage and grade information (low- and high-grade NMIBC and MIBC), which is more clinically relevant for treatment management than a classification restricted to tumor grade (low- and high-grade tumors) that was used in NCI-GWAS1 (5). Both rs8102137 and rs7257330 showed stronger association in high-grade NMIBC and MIBC categories, compared with low-grade NMIBC (Fig. 2). Because of incomplete tumor stage/grade information, this classification included 58% of all cases in our study (Supplementary Table S4). By combining the high-grade NMIBC and MIBC into one category of aggressive cancer and comparing it with nonaggressive category (low-grade NMIBC), we could classify 64% of all cases, maximizing power of the stratified analysis. It appears that both markers provided similar information for the susceptibility to...

Figure 1. Genetic and genomic landscape of the CCNE1 region. Genomic plot of the CCNE1 region is based on information from the UCSC browser (www.genome.ucsc.edu) and RNA sequencing of seven tumors and five adjacent normal bladder tissue samples. The plot shows the main CCNE1 transcripts detected by our RNA sequencing—WT1, WT2, cyclin E5, and cyclin E7. Alternative exons missing in forms cyclin E5 and cyclin E7 are marked by red boxes. Arrows indicate translation start sites and direction of protein synthesis. Genetic variants on the plot are coding nonsynonymous CCNE1 variants (marked in red), GWAS marker rs8102137, promoter variant rs7257330, an ovarian cancer candidate variant rs3218036, and several variants previously associated with various cancers (Supplementary Table S7). H3K27Ac marks in seven cell lines are a part of ENCODE track presented by the UCSC browser.

Figure 2. Forest plot for association of SNPs rs8102137 and rs7257330 in bladder cancer subgroups in the combined set of NCI-GWAS1 and NCI-GWAS2 samples. The plot shows allelic ORs and 95% CIs for SNPs. Analysis was performed only on subjects with genotype information available for both SNPs, where rs8102137 was from actual genotyping in NCI-GWAS1 and NCI-GWAS2, and rs7257330 was converted from imputed allelic dosage with >90% probability and validated by TaqMan genotyping in a subset of samples. Bladder cancer subgroups were defined in three categories (Supplementary Table S4): low-grade NMIBC (Ta with G1/G2), high-grade NMIBC (Ta with G3/G4 or all T1), and MIBC (all T2-T4; ref. 3). Incomplete clinical information category includes cases with missing tumor stage or grade data.
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Table 1. Association of CCNE1 variants with the risk of bladder cancer stratified by bladder cancer aggressiveness

<table>
<thead>
<tr>
<th>Variant</th>
<th>N (%)</th>
<th>MAF</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>Case-only MAF</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs8102137 (T/C)</td>
<td>rs7257330 (G/A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GWAS1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All controls</td>
<td>5,070</td>
<td>0.33</td>
<td>1.00 (referee)</td>
<td></td>
<td></td>
<td>0.39</td>
<td>1.00 (referee)</td>
</tr>
<tr>
<td>All cases</td>
<td>3,494</td>
<td>0.36</td>
<td>1.13 (1.05–1.20)</td>
<td>4.7 × 10^{-4}</td>
<td></td>
<td>0.41</td>
<td>1.11 (1.04–1.18)</td>
</tr>
<tr>
<td>Nonaggressive</td>
<td>1,151 (32.9)</td>
<td>0.34</td>
<td>1.07 (0.96–1.20)</td>
<td>0.21</td>
<td></td>
<td>0.13</td>
<td>0.39</td>
</tr>
<tr>
<td>Aggressive</td>
<td>1,288 (36.9)</td>
<td>0.37</td>
<td>1.18 (1.07–1.30)</td>
<td>8.8 × 10^{-4}</td>
<td></td>
<td>0.43</td>
<td>1.17 (1.07–1.29)</td>
</tr>
<tr>
<td>Missing</td>
<td>1,055 (30.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GWAS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All controls</td>
<td>5,703</td>
<td>0.32</td>
<td>1.00 (referee)</td>
<td></td>
<td></td>
<td>0.38</td>
<td>1.00 (referee)</td>
</tr>
<tr>
<td>All cases</td>
<td>2,407</td>
<td>0.35</td>
<td>1.11 (1.02–1.21)</td>
<td>0.022</td>
<td></td>
<td>0.41</td>
<td>1.10 (1.01–1.19)</td>
</tr>
<tr>
<td>Nonaggressive</td>
<td>704 (29.3)</td>
<td>0.33</td>
<td>1.00 (0.87–1.15)</td>
<td>0.97</td>
<td>0.045</td>
<td>0.39</td>
<td>1.00 (0.87–1.14)</td>
</tr>
<tr>
<td>Aggressive</td>
<td>628 (26.1)</td>
<td>0.37</td>
<td>1.16 (1.00–1.34)</td>
<td>0.046</td>
<td></td>
<td>0.43</td>
<td>1.17 (1.01–1.34)</td>
</tr>
<tr>
<td>Missing</td>
<td>1,075 (44.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GWAS1+2</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All controls</td>
<td>10,773</td>
<td>0.32</td>
<td>1.00 (referee)</td>
<td></td>
<td></td>
<td>0.39</td>
<td>1.00 (referee)</td>
</tr>
<tr>
<td>All cases</td>
<td>5,901</td>
<td>0.35</td>
<td>1.13 (1.07–1.19)</td>
<td>8.6 × 10^{-6}</td>
<td></td>
<td>0.41</td>
<td>1.11 (1.06–1.17)</td>
</tr>
<tr>
<td>Nonaggressive</td>
<td>1,855 (31.4)</td>
<td>0.33</td>
<td>1.05 (0.96–1.14)</td>
<td>0.27</td>
<td>0.013</td>
<td>0.39</td>
<td>1.01 (0.93–1.10)</td>
</tr>
<tr>
<td>Aggressive</td>
<td>1,916 (32.5)</td>
<td>0.37</td>
<td>1.18 (1.09–1.29)</td>
<td>5.6 × 10^{-5}</td>
<td></td>
<td>0.43</td>
<td>1.18 (1.09–1.27)</td>
</tr>
<tr>
<td>Missing</td>
<td>2,130 (36.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Analysis was performed on subjects with genotype information available for both SNPs, where rs8102137 was from actual genotyping in NCI-GWAS1 and NCI-GWAS2, and rs7257330 was converted from imputed allelic dosage with >90% probability. Bladder cancer aggressiveness was defined in two categories: the nonaggressive category included cases with stage Ta with grade G1/G2 tumors; the aggressive category included cases with stages T1–T4 or grade G3/G4 tumors; the missing category included cases with missing tumor stage or grade information.

MAF for rs8102137-C allele and rs7257330-A allele.

P values for estimates from multivariable logistic regression models assuming log-additive genetic effects, adjusting for age, gender, study sites, smoking status, and top eigenvectors (EV1, EV5, EV6) from principal component analysis of NCI-GWAS datasets.

P values from case-only analyses for differences of effect sizes between bladder cancer aggressiveness categories, assuming log-additive genetic effect.

aggressive bladder cancer as the ORs for both markers were attenuated to a nonsignificant level after adjustment for each other (Supplementary Table S6). However, rs7257330 provided better discrimination between aggressive and nonaggressive groups (P = 0.0015) than rs8102137 (P = 0.013) in a case-only analysis, with similar patterns of association presented in both GWAS1 and GWAS2 (Table 1). Compared with patients not carrying the rs7257330-A allele (31.7%), patients carrying one (50.6%) or two (17.7%) alleles had 27% and 34% higher risk of presenting with aggressive bladder cancer, respectively (Table 2).

Among markers with a minor allele frequency (MAF) >1%, there were no variants with strong associations independent of the original GWAS marker rs8102137 (Supplementary Table S5; Fig. 3), with the best residual association in the aggressive category being P = 0.084 for rs75996259 (r² = 0.72 with rs8102137; Supplementary Table S5). An intronic SNP rs3218036, previously reported as an ovarian cancer susceptibility candidate among Europeans (21), was one of the 47 linked variants associated with aggressive bladder cancer in our combined GWAS set [r² = 0.99 with rs8102137; OR = 1.12; 95% confidence interval (CI), 1.07–1.18; P = 1.35 × 10^{-3}]. Other CCNE1 variants previously associated with risk of breast and nasopharyngeal cancers in Asians (13, 22) were monomorphic or rare, with some suggestive associations in our study (Supplementary Tables S5 and S8). Only two rare (MAF < 0.5%) nonsynonymous coding CCNE1 variants were imputed in our dataset but with low imputation scores (0.35–0.38). One of these variants, rs61750863 (Asn260Ile), showed a suggestive independent association with aggressive bladder cancer (Supplementary Table S7). We sequenced this marker in 608 NCI-GWAS1 samples but found poor concordance between imputed and sequenced data (Supplementary Table S7); hence, this variant was not studied further.

Analysis of CCNE1 mRNA and protein expression
To explore if the CCNE1 splicing diversity could be functionally relevant for the genetic association within this
variants (Fig. 4). Expression was observed in carriers of risk alleles of both
CCNE1 genotypes. For rs8102137, NR (nonrisk homozygotes) = TT; Het = TC; R (risk homozygotes) = CC. For rs7257330, NR = GG; Het = GA; R = AA. Bladder cancer aggressiveness was defined in two categories: the nonaggressive category included cases with stage Ta with grade
1/2 tumors; the aggressive category included cases with stage T1–T4 or grade G3/G4 tumors.

**Table 2. Association of CCNE1 variants with the risk of bladder cancer under genotypic effect, stratified by bladder cancer aggressiveness**

<table>
<thead>
<tr>
<th>GWAS 1–2</th>
<th>NR, n (%)</th>
<th>Het, n (%)</th>
<th>R, n (%)</th>
<th>Het OR (95% CI)</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hom OR (95% CI)</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs8102137 (T/C)</td>
<td>All controls</td>
<td>4,957 (46.0)</td>
<td>4,657 (43.2)</td>
<td>1,159 (10.8)</td>
<td>1.00 (referent)</td>
<td>—</td>
<td>1.00 (referent)</td>
</tr>
<tr>
<td></td>
<td>All cases</td>
<td>2,461 (41.7)</td>
<td>2,710 (45.9)</td>
<td>730 (12.4)</td>
<td>1.14 (1.06–1.23)</td>
<td>5.1 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>1.25 (1.12–1.41)</td>
</tr>
<tr>
<td></td>
<td>Nonaggressive</td>
<td>811 (43.7)</td>
<td>849 (45.8)</td>
<td>195 (10.5)</td>
<td>1.09 (0.97–1.23)</td>
<td>0.15</td>
<td>1.06 (0.87–1.28)</td>
</tr>
<tr>
<td></td>
<td>Aggressive</td>
<td>765 (39.9)</td>
<td>901 (47.0)</td>
<td>250 (13.1)</td>
<td>1.22 (1.08–1.37)</td>
<td>9.1 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>1.35 (1.14–1.61)</td>
</tr>
<tr>
<td>rs7257330 (G/A)</td>
<td>All controls</td>
<td>4,092 (38.0)</td>
<td>5,046 (46.8)</td>
<td>1,635 (15.2)</td>
<td>1.00 (referent)</td>
<td>—</td>
<td>1.00 (referent)</td>
</tr>
<tr>
<td></td>
<td>All cases</td>
<td>2,014 (34.1)</td>
<td>2,900 (45.9)</td>
<td>987 (16.7)</td>
<td>1.15 (1.06–1.24)</td>
<td>3.8 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>1.21 (1.09–1.34)</td>
</tr>
<tr>
<td></td>
<td>Nonaggressive</td>
<td>681 (36.7)</td>
<td>902 (48.6)</td>
<td>272 (14.7)</td>
<td>1.07 (0.95–1.22)</td>
<td>0.26</td>
<td>0.99 (0.83–1.18)</td>
</tr>
<tr>
<td></td>
<td>Aggressive</td>
<td>607 (31.7)</td>
<td>970 (50.6)</td>
<td>339 (17.7)</td>
<td>1.27 (1.12–1.43)</td>
<td>1.2 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>1.34 (1.14–1.57)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Analysis was performed only on subjects with genotype information available for both SNPs, where rs8102137 was from actual genotyping in NCI-GWAS1 and NCI-GWAS2, and rs7257330 was converted from imputed allelic dosage with >0.9 probability. For rs8102137, NR (nonrisk homozygotes) = TT; Het = TC; R (risk homozygotes) = CC. For rs7257330, NR = GG; Het = GA; R = AA.

<sup>b</sup>For rs7257330, NR (nonrisk homozygotes) = TT; Het = TC; R (risk homozygotes) = CC. For rs7257330, NR = GG; Het = GA; R = AA.

We evaluated mRNA expression of each CCNE1 transcript in 41 bladder tumors and 40 adjacent normal bladder tissues (Supplementary Table 2). Total CCNE1 mRNA expression (all splicing forms) and expression of the cyclin E<sub>S</sub> transcript were increased in tumors compared with normal tissues (3.68-fold, P = 5.81 × 10<sup>−12</sup> and 3.56-fold, P = 1.40 × 10<sup>−9</sup>, respectively), but were not associated with rs8102137 and rs7257330 genotypes. Cyclin E<sub>r</sub> transcript levels were similar in normal and tumor tissues (1.19-fold, P = .50), but higher expression was observed in carriers of risk alleles of both variants (Fig. 4).

On the basis of mRNA expression levels, we estimated that the cyclin E<sub>S</sub> and E<sub>r</sub> are minor alternative forms, representing only 8% and 1%–2% of total CCNE1 expression, respectively. We used IHC analysis to measure total cyclin E protein expression in bladder tissues, reasoning that this analysis would mostly detect the two major WT-cyclin E isoforms, but not the minor isoforms. Consistent with mRNA expression, cyclin E protein expression was stronger in tumors compared with adjacent normal tissues (Fig. 5A). In a large set of well-characterized bladder tumors (n = 265), nuclear cyclin E expression, scored on the scale of 0 to 6 (Fig. 5B), was increased in aggressive tumors (P = .013; Fig. 5C), but was not associated with age, gender, and smoking (Supplementary Table S8). In multivariable linear regression models additionally adjusted for the categories of bladder cancer aggressiveness, the risk alleles of both markers (rs8102137 and rs7257330) were associated with increased nuclear cyclin E expression, with P = 0.024 for rs7257330 and P = 0.078 for rs8102137 (Fig. 5C and Supplementary Table S8). Some cytoplasmic cyclin E expression was also detectable in bladder tumors but was not associated with the variables examined (Supplementary Tables S8).

**Functional effects of cyclin E overexpression**

For further functional evaluation, we cloned the WT1, WT2, and the cyclin E<sub>r</sub> isoforms, because mRNA expression of these forms was associated with CCNE1 genotypes in our experiments described above. We transiently transfected corresponding expression constructs into human cells HeLa and HTB5 to produce specific exogenous recombinant cyclin E protein isoforms. Because these cell lines endogenously express cyclin E, the exogenous cyclin E forms were specifically detected with an antibody for a Halo-tag protein present on all recombinant but not on the endogenously expressed forms. In both cell lines, confocal imaging of recombinant proteins produced by CCNE1 expression constructs showed nuclear expression of the WT isoforms (WT1 and WT2), in agreement with endogenous cyclin E expression observed in the same cell lines. In contrast, the
recombinant cyclin E_T isoform was expressed in the cytoplasm (Supplementary Fig. S3A).

Cyclin E mostly functions as a complex with CDK2, which is formed in the cytoplasm, but then shuffles to the nucleus where it regulates the cell-cycle progression (26, 27). We used in situ protein interaction proximity ligation assays (28) to evaluate this critical function of cyclin E isoforms in HTB5 cells (Supplementary Fig. S3B). Compared with endogenous cyclin E, both recombinant WT forms showed a similar ability to interact with endogenous CDK2. However, the recombinant cyclin E_T isoform showed statistically significantly decreased nuclear interaction with CDK2 (Supplementary Fig. S3C). To explore the effects of recombinant cyclin E isoforms on cell cycle, HeLa and HTB5 cells were first synchronized at the G0–G1 checkpoint and then transiently transfected to produce the specific cyclin E isoforms. The effects of cyclin E recombinant forms on the cell cycle were measured as a decrease in the percentages of cells accumulated at G0–G1 stage, which is indicative of cell-cycle progression. WT1 and WT2 forms, which showed strong interaction with CDK2 in the previous experiment, also caused significant cell-cycle progression, whereas the cyclin E_T form demonstrated a decreased ability to promote cell-cycle progression, consistent with its inability to sufficiently interact with CDK2 (Supplementary Fig. S3D).

Discussion

The initial association of the CCNE1 region with high-grade bladder cancer was identified by our NCI-GWAS1 for a marker rs8102137 (5). We now explored this GWAS signal in the combined set of NCI-GWAS1 and NCI-GWAS2 (6), which included 5,942 bladder cancer cases and 10,857 controls. We used a clinical classification based on combined stage and grade information, with high-grade NMIBC and MIBC being classified as aggressive and low-grade NMIBC being classified as nonaggressive cancers. After detailed fine-mapping analysis of the region, we report that the original GWAS marker rs8102137 represents a group of 47 linked most associated markers, and the association of these markers was limited to aggressive bladder cancer cases. This pattern of association was comparable in both GWAS sets (Table 1 and 2), although the association with bladder cancer risk overall was only borderline significant in NCI-GWAS2. This could be due to a smaller proportion of aggressive bladder cancer cases in NCI-GWAS2 (26.1%) compared with NCI-GWAS 1 (36.9%; Table 1).

The exact functional variant(s) among the set of the 47 linked markers in the CCNE1 region may be difficult to pinpoint. However, we propose that the promoter variant rs7257330 represents a group of 47 linked most associated markers, and the association of these markers was limited to aggressive bladder cancer cases. This pattern of association was comparable in both GWAS sets (Table 1 and 2), although the association with bladder cancer risk overall was only borderline significant in NCI-GWAS2. This could be due to a smaller proportion of aggressive bladder cancer cases in NCI-GWAS2 (26.1%) compared with NCI-GWAS 1 (36.9%; Table 1).

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progression generates cells with genomic alterations (aneuploid or polyploid cells; refs. 29, 30) and may further the development of aggressive cancer. The second mechanism could be related to the increased mRNA expression of a minor alternative splicing form, cyclin ET. We found that cyclin ET is expressed in the cytoplasm and has poor ability to interact with CDK2 and promote the cell cycle. However, the cyclin ET isoform lacks the CLS, which is necessary for correct cellular segregation (24, 25); hence, its increased expression may also contribute to genomic instability (31).

Limitations of this study include the need of validation of rs7257330 as a prognostic marker for bladder cancer. Only patients of European ancestry were involved in our study, and validation studies in patients from other population groups are needed before generalizing our conclusions.

Information on environmental, epidemiologic, genetic, and modifiable factors, such as family and infection history, alcohol and tobacco use, and genotypes of relevant germline and somatic genetic variants, can be combined to develop clinically useful risk prediction models. Each of these factors may be only weakly or moderately informative when considered individually. However, a combination of these factors could improve the utility of prediction models and help in risk assessment, early detection and screening, therapeutic response prediction, prognosis, and survivorship. As the first and the only germline genetic marker with statistically significant discrimination between aggressive and nonaggressive bladder cancer in two large GWAS, the CCNE1 genetic variant rs7257330 represents a promising candidate for the inclusion into risk prediction models.
Disclosure of Potential Conflicts of Interest

G.L. Andriole Jr. is a consultant/advisory board member for Augmenix, Bayer, Genomic Health, GlaxoSmithKline. Myriad Genetics. B. Ljungberg is a consultant/advisory board member for Pfizer, Novartis, and GlaxoSmithKline. H.B. Grossman has ownership interest (including patents) in Abbott Molecular and is a consultant/board member for Nucleix. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Analysis of cyclin E expression in bladder tissues. A, images of cyclin E expression in one pair of tumor-adjacent normal bladder tissue samples, representative of eight pairs tested. Immunostaining was done with an anti-CCNE1 rabbit polyclonal antibody (HPA018169; Sigma; dilution 1:85). Images are presented at ×10 magnification with 0.1 mm scale bars. B, representative images of bladder tumors with nuclear cyclin E expression scores of 0, 2, 4, and 6, which is a sum of nuclear positivity (scored 0–3) and intensity (scored 0–3). The samples are a part of a TMA that includes 265 bladder tumors; immunostaining was done with an anti-CCNE1 rabbit monoclonal antibody (1655-1; Epitomics; dilution 1:250). C, association between IHC cyclin E nuclear scores in 265 bladder tumors, bladder cancer aggressiveness, and genotypes of rs8102137 and rs7257330. The scores are shown as least squares mean values with 95% CIs, based on multivariable linear regression models assuming additive effects of SNPs, with adjustment for age, gender, two study subsites, smoking, and categories of tumor aggressiveness, when appropriate, nonaggressive and aggressive.
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Study supervision: M.R. Karagas, X. Wu, B. Ljungberg, M. Dorrorsoro, M.C. Pike, N. Rothman, L. Prokunina-Olsson

Other (electrophoretic mobility shift assays): B. Muchmore

Other (IRB work, amendment to approve use of tissue for molecular studies): M. Schwenner

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