

Cancer Research



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

Yi-Ping Fu, Indu Kohaar, Lee E. Moore, et al.

Cancer Res 2014;74:5808-5818.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/74/20/5808>

Cited Articles This article cites by 31 articles, 16 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/74/20/5808.full.html#ref-list-1>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

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

Yi-Ping Fu¹, Indu Kohaar¹, Lee E. Moore², Petra Lenz³, Jonine D. Figueroa², Wei Tang¹, Patricia Porter-Gill¹, Nilanjan Chatterjee², Alexandra Scott-Johnson¹, Montserrat Garcia-Closas⁴, Brian Muchmore¹, Dalsu Baris², Ashley Paquin¹, Kris Ylaya⁵, Molly Schwenn⁶, Andrea B. Apolo⁷, Margaret R. Karagas⁸, McAnthony Tarway¹, Alison Johnson⁹, Adam Mumy¹, Alan Schned⁸, Liliana Guedez¹⁰, Michael A. Jones¹¹, Masatoshi Kida¹², GM Monawar Hosain¹³, Nuria Malats¹⁴, Manolis Kogevinas^{15,16,17,18}, Adonina Tardon^{18,19}, Consol Serra^{18,20}, Alfredo Carrato²¹, Reina Garcia-Closas²², Josep Lloreta²³, Xifeng Wu²⁴, Mark Purdue², Gerald L. Andriole Jr²⁵, Robert L. Grubb III²⁵, Amanda Black², Maria T. Landi², Neil E. Caporaso², Paolo Vineis^{26,27}, Afshan Siddiq²⁶, H. Bas Bueno-de-Mesquita^{28,29,30,31}, Dimitrios Trichopoulos^{32,33,34}, Börje Ljungberg³⁵, Gianluca Severi^{27,36,37}, Elisabete Weiderpass^{38,39,40,41}, Vittorio Krogh⁴², Miren Dorronsoro⁴³, Ruth C. Travis⁴⁴, Anne Tjønneland⁴⁵, Paul Brennan⁴⁶, Jenny Chang-Claude⁴⁷, Elio Riboli²⁶, Jennifer Prescott^{48,49}, Constance Chen⁴⁹, Immaculata De Vivo^{48,49}, Edward Giovannucci⁵⁰, David Hunter⁴⁹, Peter Kraft⁴⁹, Sara Lindstrom⁴⁹, Susan M. Gapstur⁵¹, Eric J. Jacobs⁵¹, W. Ryan Diver⁵¹, Demetrius Albanes², Stephanie J. Weinstein², Jarmo Virtamo⁵², Charles Kooperberg⁵³, Chancellor Hohensee⁵³, Rebecca J. Rodabough⁵³, Victoria K. Cortessis^{54,55}, David V. Conti⁵⁵, Manuela Gago-Dominguez⁵⁶, Mariana C. Stern⁵⁵, Malcolm C. Pike⁵⁷, David Van Den Berg⁵⁵, Jian-Min Yuan⁵⁸, Christopher A. Haiman⁵⁵, Olivier Cussenot^{59,60,61}, Geraldine Cancel-Tassin^{60,61}, Morgan Roupret^{60,61,62}, Eva Comperat^{60,61,62}, Stefano Porru⁶³, Angela Carta⁶³, Sofia Pavanello⁶⁴, Cecilia Arici⁶³, Giuseppe Mastrangelo⁶⁴, H. Barton Grossman⁶⁵, Zhaoming Wang⁶⁶, Xiang Deng⁶⁶, Charles C. Chung⁶⁶, Amy Hutchinson⁶⁶, Laurie Burdette⁶⁶, William Wheeler⁶⁷, Joseph Fraumeni Jr², Stephen J. Chanock², Stephen M. Hewitt⁵, Debra T. Silverman², Nathaniel Rothman², and Ludmila Prokunina-Olsson¹

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 $r^2 \geq 0.7$) 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 only 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. *Cancer Res*; 74(20); 5808–18. ©2014 AACR.

¹Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. ²Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. ³Clinical Research Directorate/Clinical Monitoring Research Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland. ⁴Division of Genetics and Epidemiology, Institute of Cancer Research, London, United Kingdom. ⁵Laboratory of Pathology, Center of Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. ⁶Maine Cancer Registry, Augusta, Maine. ⁷Genitourinary Malignancy Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland. ⁸Geisel School

of Medicine at Dartmouth, Lebanon, New Hampshire. ⁹Vermont Cancer Registry, Burlington, Vermont. ¹⁰Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. ¹¹Department of Pathology and Laboratory Medicine, Maine Medical Center, Portland, Maine. ¹²Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont. ¹³New Hampshire State Cancer Registry, Concord, New Hampshire. ¹⁴Spanish National Cancer Research Centre, Madrid, Spain. ¹⁵Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain. ¹⁶Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain. ¹⁷National School of Public Health, Athens, Greece. ¹⁸CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, Spain. ¹⁹Instituto Universitario de

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 genomically stable, whereas high-grade NMIBC and MIBC classified as aggressive and genomically 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-GWAS1; 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 from G1 (preparation for DNA replication) to S (DNA synthesis) phase 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.

Oncología, Universidad de Oviedo, Oviedo, Spain. ²⁰Universitat Pompeu Fabra, Barcelona, Spain. ²¹Ramón y Cajal Hospital, Madrid, Spain. ²²Unidad de Investigación, Hospital Universitario de Canarias, La Laguna, Spain. ²³Hospital del Mar-IMIM, Universitat Pompeu Fabra, Barcelona, Spain. ²⁴Department of Epidemiology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas. ²⁵Division of Urologic Surgery, Washington University School of Medicine, St. Louis, Missouri. ²⁶School of Public Health, Imperial College London, London, United Kingdom. ²⁷Human Genetics Foundation (HuGeF), Torino, Italy. ²⁸National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands. ²⁹Department of Gastroenterology and Hepatology, University Medical Centre, Utrecht, the Netherlands. ³⁰Department of Epidemiology and Biostatistics, The School of Public Health, Imperial College London, London, United Kingdom. ³¹Department of Social and Preventive Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. ³²Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts. ³³Bureau of Epidemiologic Research, Academy of Athens, Athens, Greece. ³⁴Hellenic Health Foundation, Athens, Greece. ³⁵Department of Surgical and Perioperative Sciences, Urology and Andrology, Umeå University, Umeå, Sweden. ³⁶Cancer Epidemiology Centre, Cancer Council Victoria, Melbourne, Australia. ³⁷Centre for Epidemiology and Biostatistics, University of Melbourne, Australia. ³⁸Department of Community Medicine, Faculty of Health Sciences, University of Tromsø, The Arctic University of Norway, Tromsø, Norway. ³⁹Department of Research, Cancer Registry of Norway, Oslo, Norway. ⁴⁰Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden. ⁴¹Samfundet Folkhälsan, Helsinki, Finland. ⁴²Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy. ⁴³Public Health Division of Gipuzkoa, Basque Regional Health Department and Ciberesp-Biodonostia, San Sebastian, Spain. ⁴⁴Cancer Epidemiology Unit, University of Oxford, Oxford, United Kingdom. ⁴⁵Danish Cancer Society Research Center, Copenhagen, Denmark. ⁴⁶International Agency for Research on Cancer, Lyon, France. ⁴⁷Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany. ⁴⁸Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. ⁴⁹Program in Genetic Epidemiology and Statistical Genetics, Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts. ⁵⁰Department of Nutrition and Epidemiology, Harvard School of Public Health, Boston, Massachusetts. ⁵¹Epidemiology

Research Program, American Cancer Society, Atlanta, Georgia. ⁵²National Institute for Health and Welfare, Helsinki, Finland. ⁵³Fred Hutchinson Cancer Research Center, Division of Public Health Sciences, Seattle, Washington. ⁵⁴Department of Obstetrics and Gynecology, Keck School of Medicine of USC, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California. ⁵⁵Department of Preventive Medicine, Keck School of Medicine of USC, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California. ⁵⁶Genomic Medicine Group, Galician Foundation of Genomic Medicine, Complejo Hospitalario Universitario de Santiago, Servicio Galego de Saude (SERGAS), Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain. ⁵⁷Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, New York. ⁵⁸University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania. ⁵⁹AP-HP, Hopital Tenon, GHU-Est, Department of Urology, Paris, France. ⁶⁰Centre de Recherche sur les Pathologies Prostatiques, Paris, France. ⁶¹UPMC Univ Paris 06, ONCOTYPE-URO, Paris, France. ⁶²AP-HP, Hopital Pitie-Salpetriere, GHU-Est, Departments of Urology and Pathology, Paris, France. ⁶³Department of Medical and Surgical Specialties, Radiological Sciences and Public Health, University of Brescia, Brescia, Italy. ⁶⁴Department of Cardiac, Thoracic and Vascular Sciences, University of Padova, Padua, Italy. ⁶⁵Department of Urology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas. ⁶⁶Cancer Genomics Research Laboratory, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland. ⁶⁷Information Management Services, Rockville, Maryland.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Y.-P. Fu and I. Kohaar contributed equally to this article.

Corresponding Author: Ludmila Prokunina-Olsson, Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, 8717 Grovemont Circle, Bethesda 20892-4605 MD. Phone: 301-443-5297; Fax: 301-443-3234; E-mail: prokuninal@mail.nih.gov

doi: 10.1158/0008-5472.CAN-14-1531

©2014 American Association for Cancer Research.

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 substudies. 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' 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

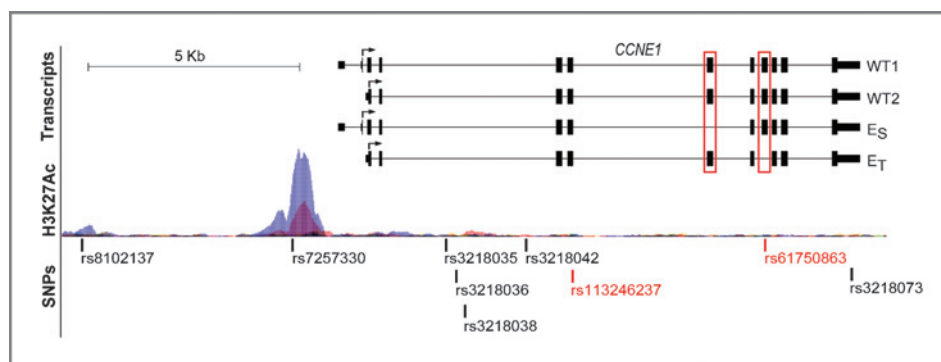


Figure 1. Genetic and genomic landscape of the *CCNE1* region. Genomic plot of the *CCNE1* region is based on information from the UCSC browser (www.genome.ucsc.edu) and RNA sequencing of seven tumors and five adjacent normal bladder tissue samples. The plot shows the main *CCNE1* transcripts detected by our RNA sequencing—*WT1*, *WT2*, *cyclin Es*, and *cyclin Et*. Alternative exons missing in forms *cyclin Es* and *cyclin Et* 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.

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

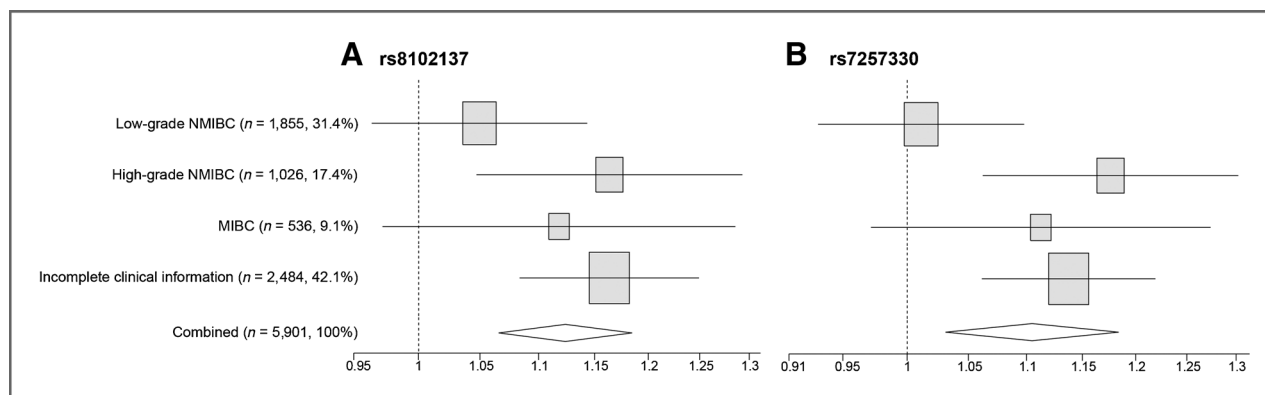


Figure 2. Forest plot for association of SNPs rs8102137 and rs7257330 in bladder cancer subgroups in the combined set of NCI-GWAS1 and NCI-GWAS2 samples. The plot shows allelic ORs and 95% CIs for SNPs. Analysis was performed only on subjects with genotype information available for both SNPs, where rs8102137 was from actual genotyping in NCI-GWAS1 and NCI-GWAS2, and rs7257330 was converted from imputed allelic dosage with $\geq 90\%$ 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.

Table 1. Association of *CCNE1* variants with the risk of bladder cancer stratified by bladder cancer aggressiveness

| | <i>N</i> (%) ^a | MAF ^b | OR (95% CI) ^c | <i>P</i> ^c | Case-only <i>P</i> ^d | MAF ^b | OR (95% CI) ^c | <i>P</i> ^c | Case-only <i>P</i> ^d |
|----------------|---------------------------|------------------|--------------------------|-----------------------|------------------------------------|------------------|--------------------------|-----------------------|------------------------------------|
| | | | rs8102137 (T/C) | | | | rs7257330 (G/A) | | |
| GWAS1 | | | | | | | | | |
| All controls | 5,070 | 0.33 | 1.00 (referent) | — | — | 0.39 | 1.00 (referent) | — | — |
| All cases | 3,494 | 0.36 | 1.13 (1.05–1.20) | 4.7×10^{-4} | — | 0.41 | 1.11 (1.04–1.18) | 0.0022 | — |
| Nonaggressive | 1,151 (32.9) | 0.34 | 1.07 (0.96–1.20) | 0.21 | 0.13 | 0.39 | 1.01 (0.91–1.13) | 0.80 | 0.031 |
| Aggressive | 1,288 (36.9) | 0.37 | 1.18 (1.07–1.30) | 8.8×10^{-4} | — | 0.43 | 1.17 (1.07–1.29) | 8.4×10^{-4} | — |
| Missing | 1,055 (30.2) | — | — | — | — | — | — | — | — |
| GWAS2 | | | | | | | | | |
| All controls | 5,703 | 0.32 | 1.00 (referent) | — | — | 0.38 | 1.00 (referent) | — | — |
| All cases | 2,407 | 0.35 | 1.11 (1.02–1.21) | 0.022 | — | 0.41 | 1.10 (1.01–1.19) | 0.026 | — |
| Nonaggressive | 704 (29.3) | 0.33 | 1.00 (0.87–1.15) | 0.97 | 0.045 | 0.39 | 1.00 (0.87–1.14) | 1.00 | 0.027 |
| Aggressive | 628 (26.1) | 0.37 | 1.16 (1.00–1.34) | 0.046 | — | 0.43 | 1.17 (1.01–1.34) | 0.031 | — |
| Missing | 1,075 (44.7) | — | — | — | — | — | — | — | — |
| GWAS1+2 | | | | | | | | | |
| All controls | 10,773 | 0.32 | 1.00 (referent) | — | — | 0.39 | 1.00 (referent) | — | — |
| All cases | 5,901 | 0.35 | 1.13 (1.07–1.19) | 8.6×10^{-6} | — | 0.41 | 1.11 (1.06–1.17) | 6.0×10^{-5} | — |
| Nonaggressive | 1,855 (31.4) | 0.33 | 1.05 (0.96–1.14) | 0.27 | 0.013 | 0.39 | 1.01 (0.93–1.10) | 0.79 | 0.0015 |
| Aggressive | 1,916 (32.5) | 0.37 | 1.18 (1.09–1.28) | 5.6×10^{-5} | — | 0.43 | 1.18 (1.09–1.27) | 4.7×10^{-5} | — |
| Missing | 2,130 (36.1) | — | — | — | — | — | — | — | — |

^aAnalysis 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 $\geq 90\%$ 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.

^bMAF for rs8102137-C allele and rs7257330-A allele.

^c*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.

^d*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 ($r^2 = 0.72$ 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 [$r^2 = 0.99$ with rs8102137; OR = 1.12; 95% confidence interval (CI), 1.07–1.18; $P = 1.35 \times 10^{-5}$]. 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

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

| GWAS 1+2 | NR, n (%) ^a | Het, n (%) ^a | R, n (%) ^a | Het OR (95% CI) ^b | P ^b | Hom OR (95% CI) ^b | P ^b |
|-----------------|------------------------|-------------------------|-----------------------|------------------------------|----------------------|------------------------------|----------------------|
| rs8102137 (T/C) | | | | | | | |
| All controls | 4,957 (46.0) | 4,657 (43.2) | 1,159 (10.8) | 1.00 (referent) | — | 1.00 (referent) | — |
| All cases | 2,461 (41.7) | 2,710 (45.9) | 730 (12.4) | 1.14 (1.06–1.23) | 5.1×10^{-4} | 1.25 (1.12–1.41) | 1.4×10^{-4} |
| Nonaggressive | 811 (43.7) | 849 (45.8) | 195 (10.5) | 1.09 (0.97–1.23) | 0.15 | 1.06 (0.87–1.28) | 0.58 |
| Aggressive | 765 (39.9) | 901 (47.0) | 250 (13.1) | 1.22 (1.08–1.37) | 9.1×10^{-4} | 1.35 (1.14–1.61) | 7.0×10^{-4} |
| rs7257330 (G/A) | | | | | | | |
| All controls | 4,092 (38.0) | 5,046 (46.8) | 1,635 (15.2) | 1.00 (referent) | — | 1.00 (referent) | — |
| All cases | 2,014 (34.1) | 2,900 (49.1) | 987 (16.7) | 1.15 (1.06–1.24) | 3.8×10^{-4} | 1.21 (1.09–1.34) | 4.1×10^{-4} |
| Nonaggressive | 681 (36.7) | 902 (48.6) | 272 (14.7) | 1.07 (0.95–1.22) | 0.26 | 0.99 (0.83–1.18) | 0.90 |
| Aggressive | 607 (31.7) | 970 (50.6) | 339 (17.7) | 1.27 (1.12–1.43) | 1.2×10^{-4} | 1.34 (1.14–1.57) | 3.7×10^{-4} |

^aAnalysis 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. 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.

^bP 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.

region, we performed RNA sequencing in tumors and adjacent normal bladder tissues. In addition to two wild-type (WT) forms encoded from translation start sites within the first (*WT1*) or second exon (*WT2*), we detected two transcripts (23, 24) encoding aberrant forms of cyclin E (cyclin E_S and cyclin E_T) with mutually exclusive deletions of internal exons 5 or 7 (Fig. 1). The sequences of the full-length transcripts for the alternative *CCNE1* forms were deposited to NCBI GenBank. The cyclin E_S form (GenBank KF672848) lacks the CDK2-binding region, eliminated by an in-frame exclusion of 49 aa (147 bp, exon 5; ref. 23) within the cyclin box domain. The cyclin E_T form (GenBank KF672847) has an in-frame exclusion of 45 aa (135 bp, exon 7; ref. 24), which eliminates the centrosomal localization signal (CLS; Supplementary Fig. S2), which is critical for DNA synthesis and correct cellular segregation (25).

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_S* transcript were increased in tumors compared with normal tissues (3.68-fold, $P = 5.81 \times 10^{-12}$ and 3.56-fold, $P = 1.40 \times 10^{-9}$, respectively), but were not associated with rs8102137 and rs7257330 genotypes. *Cyclin E_T* 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_S* and *E_T* 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 alternative 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_T 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 cells 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

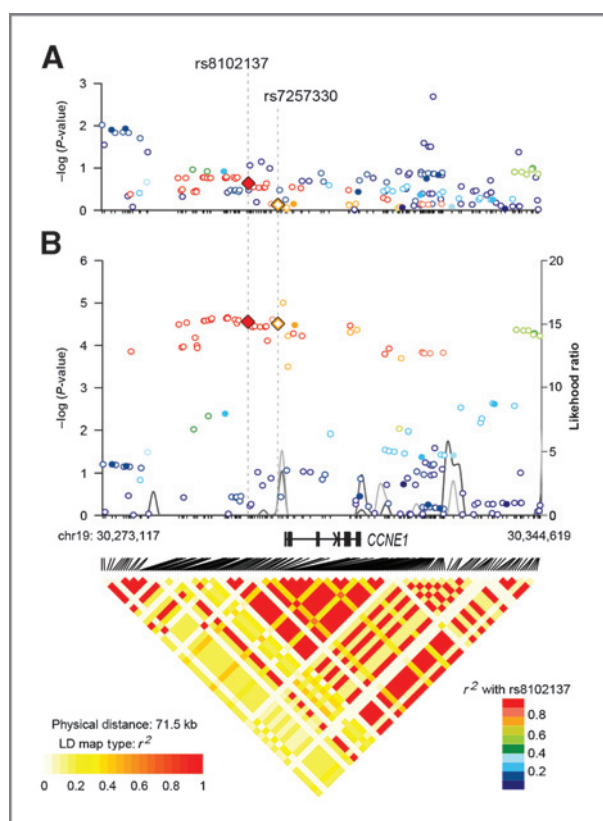


Figure 3. Association results, LD (r^2), and recombination plots of the *CCNE1* region in combined NCI-GWAS1 and NCI-GWAS2 samples. Left y-axis ($-\log_{10}$ scale) presents P values for GWAS (filled circles) and well-imputed markers (IMPUTE2 score ≥ 0.9 , open circles), adjusted for age, gender, study sites, smoking status (ever/never), and top eigenvectors (EV1, EV5, EV6) from principal component analysis. The color scheme is based on r^2 values between rs8102137 and corresponding markers. Right y-axis presents likelihood ratio of putative recombination hotspots based on five sets of 100 randomly selected controls from NCI-GWAS1 and shown as connected gray lines. The GWAS marker rs8102137 and the promoter variant rs7257330 are shown as diamonds. Pair-wise r^2 values based on all control samples are displayed at the bottom of the plot for all 165 SNPs included in the analyses. Genomic coordinates are based on the NCBI Human Genome Build 37.1/UCSC hg19 assembly. A, association analysis in a subset of patients with nonaggressive bladder cancer (stage Ta with grade G1 or G2, $n = 1,870$) versus all controls ($n = 10,857$). B, association analysis in a subset of patients with aggressive bladder cancer (stage Ta with grade G3+ or stages T1–T4 with any grade, $n = 1,930$) versus all controls ($n = 10,857$).

recombinant cyclin E_T form 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 statisti-

cally 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).

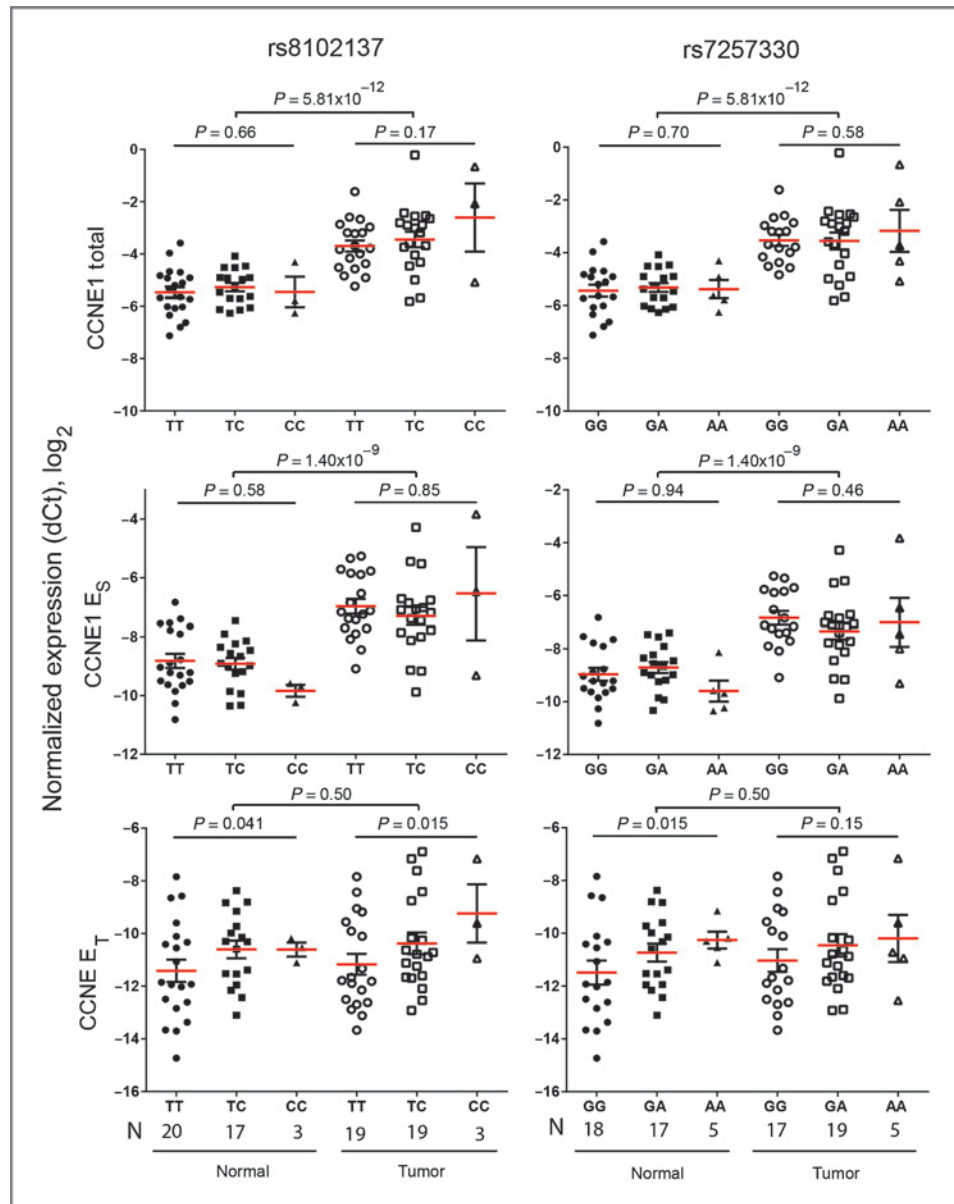
Discussion

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-GWAS1 (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 appears to be a good representative of this group and also a functional variant, which showed strong association with nuclear expression of the cyclin E protein in bladder tumors, and exhibited allele-specific interaction with nuclear proteins in various cell lines. The location of rs7257330 within a region of a uniquely strong H3K27Ac marks characteristic of active enhancers (Fig. 1) also suggests that rs7257330 may function as a regulatory variant within the *CCNE1* promoter.

Nonaggressive bladder tumors are considered to be genomically stable in contrast to genomically unstable aggressive tumors (3, 4). On the basis of our genetic and functional studies, we suggest that cyclin E may contribute to the genomic instability and aggressive bladder cancer through at least two possible functional mechanisms. The first mechanism could be related to an rs7257330 genotype-specific increase in nuclear expression of cyclin E protein in bladder tumors. The majority of detectable cyclin E protein is represented by the WT cyclin E isoforms, which we showed to be functional in interaction with CDK2, and the ability to promote the cell cycle. Rapid cell-cycle

Figure 4. Analysis of *CCNE1* mRNA expression in bladder tumors and adjacent normal tissue and in relation to rs8102137 and rs7257330 genotypes. Expression of total *CCNE1* (all transcripts), *cyclin E_S*, and *cyclin E_T* transcripts was measured with TaqMan assays. *CCNE1* expression was normalized to a geometric mean of endogenous controls (*B2M*, *GAPDH*, and *PPIA*) and presented on the log₂ scale. Less-negative values correspond to higher relative expression of *CCNE1* transcripts. The association between normalized *CCNE1* mRNA expression and risk allele counts (0, 1, and 2) of both SNPs was evaluated with multivariable linear regression models, adjusting for age and gender. Most of bladder tumors used for expression analysis (30 of 41 tumors) are of aggressive type (Supplementary Table S2).



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 E_T*. We found that *cyclin E_T* is expressed in the cytoplasm and has poor ability to interact with CDK2 and promote the cell cycle. However, the *cyclin E_T* 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.

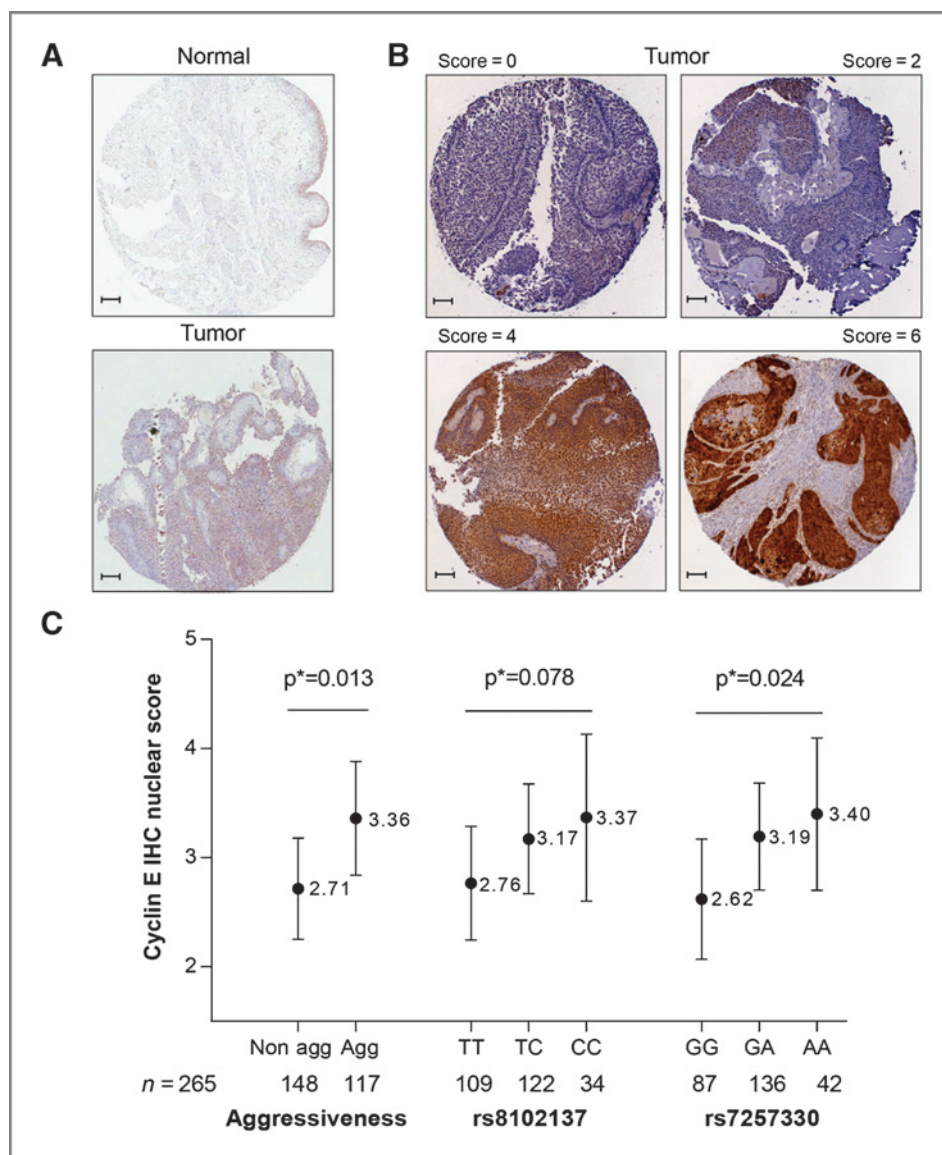


Figure 5. IHC analysis of cyclin E expression in bladder tissues. **A**, images of cyclin E expression in one pair of tumor-adjacent normal bladder tissue samples, representative of eight pairs tested. Immunostaining was done with an anti-CCNE1 rabbit polyclonal antibody (HPA018169; Sigma; dilution 1:85). Images are presented at $\times 10$ 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.

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

Conception and design: Y.-P. Fu, I. Kohaar, L.E. Moore, M. Garcia-Closas, D. Baris, A.B. Apolo, M.R. Karagas, C. Serra, P. Vineis, G. Severi, A. Tjønneland, S.J. Chanock, L. Prokunina-Olsson

Development of methodology: L.E. Moore, M.R. Karagas, A. Tardon, G. Severi, M. Gago-Dominguez, S.M. Hewitt

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Kohaar, L.E. Moore, P. Lenz, J.D. Figueroa, P. Porter-Gill, A. Scott-Johnson, D. Baris, A. Paquin, M. Schwenn, M.R. Karagas, M. Tarway, A. Johnson, A. Mumy, A. Schned, L. Guede, M.A. Jones, M. Kida, G.M.M. Hosain, N. Malats, M. Kogevinas, A. Tardon, C. Serra, A. Carrato, R. Garcia-Closas, J. Lloreta, X. Wu, M. Purdue, R.L. Grubb III, A. Black, M.T. Landi, N.E. Caporaso, A. Siddiq, H.B. Bueno-de-Mesquita, D. Trichopoulos, B. Ljungberg, G. Severi, V. Krogh, M. Dorronsoro, R.C. Travis, P. Brennan, J. Chang-Claude, J. Prescott, I.D.

Vivo, E. Giovannucci, D. Hunter, P. Kraft, S. Lindstrom, S.M. Gapstur, E.J. Jacobs, W.R. Diver, D. Albanes, S.J. Weinstein, J. Virtamo, C. Kooperberg, V.K. Cortessis, D.V. Conti, M. Gago-Dominguez, M.C. Stern, M.C. Pike, D.V.D. Berg, J.-M. Yuan, O. Cussenot, G. Cancel-Tassin, M. Roupert, E. Comperat, S. Porru, A. Carta, S. Pavanello, C. Arici, G. Mastrangelo, H.B. Grossman, L. Burdette, S.J. Chanock, S.M. Hewitt, D.T. Silverman, N. Rothman, L. Prokunina-Olsson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-P. Fu, I. Kohaar, L.E. Moore, J.D. Figueroa, W. Tang, N. Chatterjee, A. Scott-Johnson, A.B. Apolo, M.R. Karagas, A. Mumy, J. Lloreta, A. Tjønneland, C. Chen, E.J. Jacobs, C. Kooperberg, Z. Wang, X. Deng, C.C. Chung, W. Wheeler, S.J. Chanock, L. Prokunina-Olsson

Writing, review, and/or revision of the manuscript: Y.-P. Fu, I. Kohaar, L.E. Moore, J.D. Figueroa, N. Chatterjee, M. Garcia-Closas, A.B. Apolo, A. Johnson, A. Schned, N. Malats, M. Kogevinas, A. Tardon, C. Serra, A. Carrato, J. Lloreta, X. Wu, M. Purdue, G.L. Andriole Jr., R.L. Grubb III, A. Black, M.T. Landi, N.E. Caporaso, P. Vineis, H.B. Bueno-de-Mesquita, D. Trichopoulos, B. Ljungberg, G. Severi, V. Krogh, M. Dorronsoro, R.C. Travis, A. Tjønneland, J. Chang-Claude, E. Riboli, J. Prescott, I.D. Vivo, E. Giovannucci, D. Hunter, P. Kraft, S.M. Gapstur, E.J. Jacobs, W.R. Diver, D. Albanes, S.J. Weinstein, J. Virtamo, C. Kooperberg, V.K. Cortessis, M.C. Stern, J.-M. Yuan, C.A. Haiman, O. Cussenot, G. Cancel-Tassin, M. Roupert, S. Porru, A. Carta, S. Pavanello, C. Arici, G. Mastrangelo, H.B. Grossman, Z. Wang, J. Fraumeni Jr., S.J. Chanock, D.T. Silverman, N. Rothman, L. Prokunina-Olsson

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.E. Moore, D. Baris, K. Ylaja, G.M.M. Hosain, D. Albanes, C. Hohensee, R.J. Rodabough, M. Gago-Dominguez, J.-M. Yuan, G. Cancel-Tassin, X. Deng, C.C. Chung, A. Hutchinson

Study supervision: M.R. Karagas, X. Wu, B. Ljungberg, M. Dorronsoro, 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. Schwenn

Grant Support

The project was funded in whole or in part with federal funds from the intramural research program (IRP) of the NCI/NIH (contract no. HHSN261200800001E).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Additional funds to specific studies

Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (D. Albanes)—the IRP of the Division of Cancer Epidemiology and Genetics (DCEG)/NCI/NIH and by U.S. Public Health Service contracts N01-CN-45165, N01-RC-45035, N01-RC-37004, and HHSN261201000006C.

European Prospective Investigation into Cancer and Nutrition Study (P. Vineis)—ICL, Europe Against Cancer Program of the European Commission (SANCO); IARC, International Agency for Research on Cancer; France, Ligue contre le Cancer Societe 3M, Mutuelle Generale de l'Education Nationale; Institut National de la Santé et de la Recherche Médicale (INSERM); Italy, Italian Association for Research on Cancer National Research Council; Spain, Health Research Fund (FIS) of the Spanish Ministry of Health; the CIBER en Epidemiología y Salud Pública (CIBERESP), Spain; ISCIII RETIC (RD06/0020); Spanish Regional Governments of Andalusia, Asturias, Basque Country, Murcia (N 6236), and Navarra and the Catalan Institute of Oncology; UK, Cancer Research UK Medical Research Council with additional support from the Stroke Association, British Heart Foundation, Department of Health, Food Standards Agency, the

Wellcome Trust; the Netherlands, Dutch Ministry of Public Health Dutch Prevention Funds, LK Research Funds, Dutch ZON (Zorg Onderzoek Nederland), World Cancer Research Fund (WCRF); Greece, Hellenic Ministry of Health, the Stavros Niarchos Foundation and the Hellenic Health Foundation; Germany, German Cancer Aid, German Cancer Research Center Federal Ministry of Education and Research (Grant 01-EA-9401); Sweden, Swedish Cancer Society, Swedish Scientific Council, Regional Government of Skane, Sweden; and Denmark, Danish Cancer Society.

French Bladder Cancer Study (S. Benhamou)—Ligue Contre le Cancer du Val-de-Marne; Fondation de France; Groupement d'Entreprises Françaises dans la Lutte contre le Cancer; Association pour la Recherche sur le Cancer, France.

Los Angeles Bladder Cancer Study (M.C. Pike)—NIH grants R01CA65726, R01CA114665, IR01CA114665, and IP01CA86871.

New England Bladder Cancer Study (D.T. Silverman)—IRP of the DCEG/NCI/NIH and intramural contract NCI N02-CP-01037.

Nurse's Health Study and Health Professionals Follow-up Study (I.D. Vivo)—CA055075, P01 CA87969, R01 CA49449, UMI CA176726, R01 CA67262, UMI CA167552, CA082838.

Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (M. Purdue)—The NIH Genes, Environment and Health Initiative (GEI) grants HG-06-033-NCI-01 and RO1HL091172-01, U01HG004438, and NIH HHSN268200782096C.

Gene-Environment Association Studies (N. Chatterjee)—Coordination Center grant U01HG004446, the IRP of the DCEG/NCI/NIH and contracts from the Division of Cancer Prevention, NCI/NIH.

Spanish Bladder Cancer Study (D.T. Silverman)—IRP of the DCEG/NCI/NIH and intramural contract NCI N02-CP-11015. FIS/Spain 98/1274, FIS/Spain 00/0745, PI061614, and G03/174, Fundació Marató TV3, Red Temática Investigación Cooperativa en Cáncer (RTICC), Consolider ONCOBIO, EU-FP7-201663; and RO1-CA089715 and CA34627.

Women's Health Initiative (C. Kooperberg)—funds from the National Heart, Lung, and Blood Institute (NHLBI)/NIH through contracts HHSN268201100046C, HHSN268201100001C, HHSN268201100002C, HHSN268201100003C, HHSN268201100004C, and HHSN271201100004C.

Received May 28, 2014; revised August 4, 2014; accepted August 4, 2014; published online October 15, 2014.

References

- Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63:11–30.
- Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. *J Clin Oncol* 2001;19:666–75.
- Guey LT, Garcia-Closas M, Murta-Nascimento C, Lloreta J, Palencia L, Kogevinas M, et al. Genetic susceptibility to distinct bladder cancer subphenotypes. *Eur Urol* 2010;57:283–92.
- Hernandez S, Lopez-Knowles E, Lloreta J, Kogevinas M, Amorós A, Tardon A, et al. Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas. *J Clin Oncol* 2006;24:3664–71.
- Rothman N, Garcia-Closas M, Chatterjee N, Malats N, Wu X, Figueroa JD, et al. A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. *Nat Genet* 2010;42:978–84.
- Figueroa JD, Ye Y, Siddiq A, Garcia-Closas M, Chatterjee N, Prokunina-Olsson L, et al. Genome-wide association study identifies multiple loci associated with bladder cancer risk. *Hum Mol Genet* 2014;23:1387–98.
- Wu X, Ye Y, Kiemeny LA, Sulem P, Rafnar T, Matullo G, et al. Genetic variation in the prostate stem cell antigen gene PSCA confers susceptibility to urinary bladder cancer. *Nat Genet* 2009;41:991–5.
- Kiemeny LA, Thorlacius S, Sulem P, Geller F, Aben KK, Stacey SN, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. *Nat Genet* 2008;40:1307–12.
- Lew DJ, Dulic V, Reed SI. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* 1991;66:1197–206.
- Donnellan R, Chetty R. Cyclin E in human cancers. *FASEB J* 1999;13:773–80.
- Glotzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. *Nature* 1991;349:132–8.
- Nakayama N, Nakayama K, Shamima Y, Ishikawa M, Katagiri A, Iida K, et al. Gene amplification CCNE1 is related to poor survival and potential therapeutic target in ovarian cancer. *Cancer* 2010;116:2621–34.
- Liu Y, Cai H, Liu J, Fan H, Wang Z, Wang Q, et al. A miR-151 binding site polymorphism in the 3'-untranslated region of the cyclin E1 gene associated with nasopharyngeal carcinoma. *Biochem Biophys Res Commun* 2013;432:660–5.
- Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, et al. COSMIC: mining complete cancer genomes in the catalogue of somatic mutations in cancer. *Nucleic Acids Res* 2011;39:D945–50.
- Fu YP, Kohaar I, Rothman N, Earl J, Figueroa JD, Ye Y, et al. Common genetic variants in the PSCA gene influence gene expression and bladder cancer risk. *Proc Natl Acad Sci U S A* 2012;109:4974–9.
- Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature* 2012;491:56–65.
- Howie BN, Donnelly P, Marchini J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet* 2009;5:e1000529.
- Kohaar I, Porter-Gill P, Lenz P, Fu YP, Mumy A, Tang W, et al. Genetic variant as a selection marker for anti-prostate stem cell antigen immunotherapy of bladder cancer. *J Natl Cancer Inst* 2013;105:69–73.
- Lenz P, Pfeiffer R, Baris D, Schned AR, Takikita M, Poscablo MC, et al. Cell-cycle control in urothelial carcinoma: large-scale tissue array analysis of tumor tissue from Maine and Vermont. *Cancer Epidemiol Biomarkers Prev* 2012;21:1555–64.
- Bernstein BE, Birney E, Dunham I, Green ED, Gunter C, Snyder M. An integrated encyclopedia of DNA elements in the human genome. *Nature* 2012;489:57–74.
- Goode EL, Fridley BL, Vierkant RA, Cunningham JM, Phelan CM, Anderson S, et al. Candidate gene analysis using imputed genotypes:

- cell cycle single-nucleotide polymorphisms and ovarian cancer risk. *Cancer Epidemiol Biomarkers Prev* 2009;18:935–44.
22. Han JY, Wang H, Xie YT, Li Y, Zheng LY, Ruan Y, et al. Association of germline variation in CCNE1 and CDK2 with breast cancer risk, progression and survival among Chinese Han women. *PLoS One* 2012;7:e49296.
 23. Sewing A, Ronicke V, Burger C, Funk M, Muller R. Alternative splicing of human cyclin E. *J Cell Sci* 1994;107:581–8.
 24. Mumberg D, Wick M, Burger C, Haas K, Funk M, Muller R. Cyclin ET, a new splice variant of human cyclin E with a unique expression pattern during cell cycle progression and differentiation. *Nucleic Acids Res* 1997;25:2098–105.
 25. Matsumoto Y, Maller JL. A centrosomal localization signal in cyclin E required for Cdk2-independent S phase entry. *Science* 2004;306:885–8.
 26. Moore JD. In the wrong place at the wrong time: does cyclin mis-localization drive oncogenic transformation? *Nat Rev Cancer* 2013;13:201–8.
 27. Moore JD, Yang J, Truant R, Kornbluth S. Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1. *J Cell Biol* 1999;144:213–24.
 28. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* 2006;3:995–1000.
 29. Ekholm-Reed S, Mendez J, Tedesco D, Zetterberg A, Stillman B, Reed SI. Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *J Cell Biol* 2004;165:789–800.
 30. Kawamura K, Izumi H, Ma Z, Ikeda R, Moriyama M, Tanaka T, et al. Induction of centrosome amplification and chromosome instability in human bladder cancer cells by p53 mutation and cyclin E overexpression. *Cancer Res* 2004;64:4800–9.
 31. Geisen C, Moroy T. The oncogenic activity of cyclin E is not confined to Cdk2 activation alone but relies on several other, distinct functions of the protein. *J Biol Chem* 2002;277:39909–18.