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

Yi-Ping Fu[1], Indu Kohaar[1], Lee E. Moore[2], Petra Lenz[3], Jonine D. Figueroa[2], Wei Tang[1], Patricia Porter-Gill[1], Nilanjan Chatterjee[2], Alexandra Scott-Johnson[1], Montserrat Garcia-Closas[4], Brian Muchmore[1], Dalsu Baris[2], Ashley Paquin[1], Kris Ylaya[5], Molly Schwenn[6], Andrea B. Apolo[7], Margaret R. Karagas[8], McAnthony Tarway[1], Alison Johnson[9], Adam Mumy[1], Alan Schned[8], Liliana Guedez[10], Michael A. Jones[11], Masatoshi Kida[12], GM Monawar Hosain[13], Nuria Malats[14], Manolis Kogevinas[15,16,17,18], Adonina Tardon[18,19], Consol Serra[18,20], Alfredo Carrato[21], Reina Garcia-Closas[22], Josep Lloreta[23], Xifeng Wu[24], Mark Purdue[2], Gerald L. Andriole Jr[25], Robert L. Grubb III[25], Amanda Black[2], Maria T. Landi[2], Neil E. Caporaso[2], Paolo Vineis[26,27], Afshan Siddiq[26], H. Bas Bueno-de-Mesquita[28,29,30,31], Dimitrios Trichopoulos[32,33,34], Börje Ljungberg[35], Gianluca Severi[27,36,37], Elisabete Weiderpass[38,39,40,41], Vittorio Krogh[42], Miren Dorronsoro[43], Ruth C. Travis[44], Anne Tjønneland[45], Paul Brennan[46], Jenny Chang-Claude[47], Elio Riboli[26], Jennifer Prescott[48,49], Constance Chen[49], Immaculata De Vivo[48,49], Edward Govannucci[50], David Hunter[49], Peter Kraft[49], Sara Lindstrom[49], Susan M. Gapstur[51], Eric J. Jacobs[51], W. Ryan Diver[51], Demetrius Albanes[2], Stephanie J. Weinstein[2], Jarmo Virtamo[52], Charles Kooperberg[53], Chancellor Hohensee[53], Rebecca J. Rodabough[53], Victoria K. Cortessis[54,55], David V. Conti[55], Manuela Gago-Dominguez[56], Mariana C. Stern[55], Malcolm C. Pike[57], David Van Den Berg[55], Jian-Min Yuan[58], Christopher A. Haiman[55], Olivier Cussenot[59,60,61], Geraldine Cancel-Tassin[60,61], Morgan Roupret[60,61,62], Eva Comperat[60,61,62], Stefano Porru[63], Angela Carta[63], Sofia Pavanello[64], Cecilia Arici[63], Giuseppe Mastrangelo[64], H. Barton Grossman[65], Zhaoming Wang[66], Xiang Deng[66], Charles C. Chung[66], Amy Hutchinson[66], Laurie Burdette[66], William Wheeler[67], Joseph Fraumeni Jr[2], Stephen J. Chanock[2], Stephen M. Hewitt[5], Debra T. Silverman[2], Nathaniel Rothman[2], and Ludmila Prokunina-Olsson[1]

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 ($P_{trend} = 0.024$). 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.*

[1]Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. [2]Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. [3]Clinical Research Directorate/Clinical Monitoring Research Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland. [4]Division of Genetics and Epidemiology, Institute of Cancer Research, London, United Kingdom. [5]Laboratory of Pathology, Center of Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. [6]Maine Cancer Registry, Augusta, Maine. [7]Genitourinary Malignancy Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland. [8]Geisel School of Medicine at Dartmouth, Lebanon, New Hampshire. [9]Vermont Cancer Registry, Burlington, Vermont. [10]Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. [11]Department of Pathology and Laboratory Medicine, Maine Medical Center, Portland, Maine. [12]Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont. [13]New Hampshire State Cancer Registry, Concord, New Hampshire. [14]Spanish National Cancer Research Centre, Madrid, Spain. [15]Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain. [16]Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain. [17]National School of Public Health, Athens, Greece. [18]CIBER Epidemiologia y Salud Pública (CIBERESP), Barcelona, Spain. [19]Instituto 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. [20]Universitat Pompeu Fabra, Barcelona, Spain. [21]Ramón y Cajal Hospital, Madrid, Spain. [22]Unidad de Investigación, Hospital Universitario de Canarias, La Laguna, Spain. [23]Hospital del Mar-IMIM, Univesitat Pompeu Fabra, Barcelona, Spain. [24]Department of Epidemiology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas. [25]Division of Urologic Surgery, Washington University School of Medicine, St. Louis, Missouri. [26]School of Public Health, Imperial College London, London, United Kingdom. [27]Human Genetics Foundation (HuGeF), Torino, Italy. [28]National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands. [29]Department of Gastroenterology and Hepatology, University Medical Centre, Utrecht, the Netherlands. [30]Department of Epidemiology and Biostatistics, The School of Public Health, Imperial College London, London, United Kingdom. [31]Department of Social and Preventive Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. [32]Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts. [33]Bureau of Epidemiologic Research, Academy of Athens, Athens, Greece. [34]Hellenic Health Foundation, Athens, Greece. [35]Department of Surgical and Perioperative Sciences, Urology and Andrology, Umeå University, Umeå, Sweden. [36]Cancer Epidemiology Centre, Cancer Council Victoria, Melbourne, Australia. [37]Centre for Epidemiology and Biostatistics, University of Melbourne, Australia. [38]Department of Community Medicine, Faculty of Health Sciences, University of Tromsø, The Arctic University of Norway, Tromsø, Norway. [39]Department of Research, Cancer Registry of Norway, Oslo, Norway. [40]Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden. [41]Samfundet Folkhälsan, Helsinki, Finland. [42]Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy. [43]Public Health Division of Gipuzkoa, Basque Regional Health Department and Ciberesp-Biodonostia, San Sebastian, Spain. [44]Cancer Epidemiology Unit, University of Oxford, Oxford, United Kingdom. [45]Danish Cancer Society Research Center, Copenhagen, Denmark. [46]International Agency for Research on Cancer, Lyon, France. [47]Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany. [48]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [49]Program in Genetic Epidemiology and Statistical Genetics, Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts. [50]Department of Nutrition and Epidemiology, Harvard School of Public Health, Boston, Massachusetts. [51]Epidemiology Research Program, American Cancer Society, Atlanta, Georgia. [52]National Institute for Health and Welfare, Helsinki, Finland. [53]Fred Hutchinson Cancer Research Center, Division of Public Health Sciences, Seattle, Washington. [54]Department of Obstetrics and Gynecology, Keck School of Medicine of USC, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California. [55]Department of Preventive Medicine, Keck School of Medicine of USC, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California. [56]Genomic 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. [57]Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, New York. [58]University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania. [59]AP-HP, Hopital Tenon, GHU-Est, Department of Urology, Paris, France. [60]Centre de Recherche sur les Pathologies Prostatiques, Paris, France. [61]UPMC Univ Paris 06, ONCOTYPE-URO, Paris, France. [62]AP-HP, Hopital Pitie-Salpetriere, GHU-Est, Departments of Urology and Pathology, Paris, France. [63]Department of Medical and Surgical Specialties, Radiological Sciences and Public Health, University of Brescia, Brescia, Italy. [64]Department of Cardiac, Thoracic and Vascular Sciences, University of Padova, Padua, Italy. [65]Department of Urology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas. [66]Cancer Genomics Research Laboratory, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland. [67]Information Management Services, Rockville, Maryland.

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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

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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 $\geq$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 $\geq$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

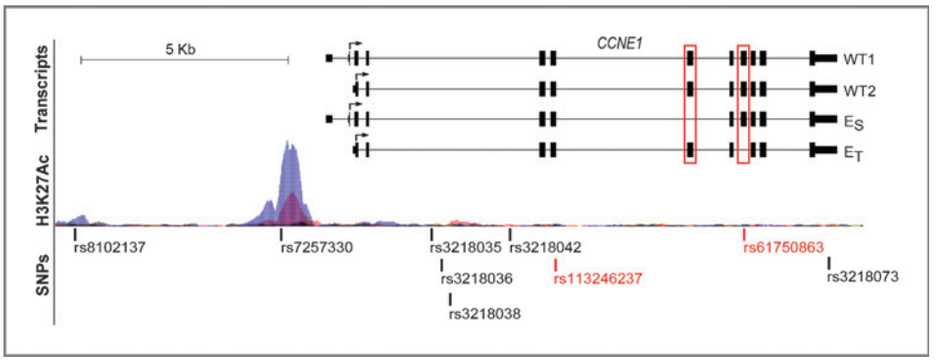

Figure 1. Genetic and genomic landscape of the *CCNE1* region. Genomic plot of the *CCNE1* region is based on information from the UCSC browser (www.genome.ucsc.edu) and RNA sequencing of seven tumors and five adjacent normal bladder tissue samples. The plot shows the main *CCNE1* transcripts detected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

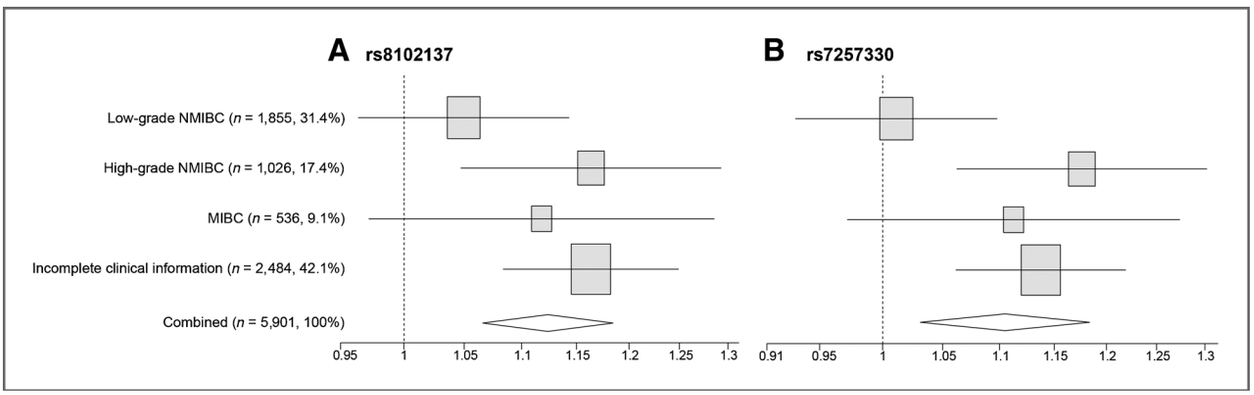

Figure 2. Forest plot for association of SNPs rs8102137 and rs7257330 in bladder cancer subgroups in the combined set of NCI-GWAS1 and NCI-GWAS2 samples. The plot shows allelic ORs and 95% CIs for SNPs. Analysis was performed only on subjects with genotype information available for both SNPs, where rs8102137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

	N (%)[a]	MAF[b]	OR (95% CI)[c]	*P*[c]	Case-only *P*[d]	MAF[b]	OR (95% CI)[c]	*P*[c]	Case-only *P*[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)								

[a]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. 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.

[b]MAF 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), 1.07–118; $P = 1.35 \times 10^{-5}$]. 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}

[a]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. 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.

[b]*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.

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

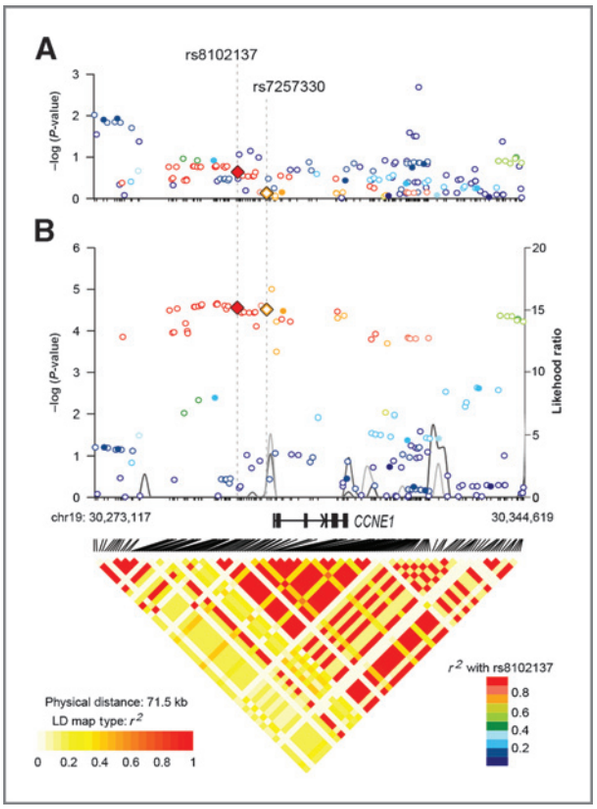

Figure 3. Association results, LD (r^2), and recombination plots of the *CCNE1* region in combined NCI-GWAS1 and NCI-GWAS2 samples. Left *y*-axis ($-\log_{10}$ scale) presents *P* values for GWAS (filled circles) and well-imputed markers (IMPUTE2 score $\geq$0.9, open circles), adjusted for age, gender, 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 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 G_0–G_1 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 G_0–G_1 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 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

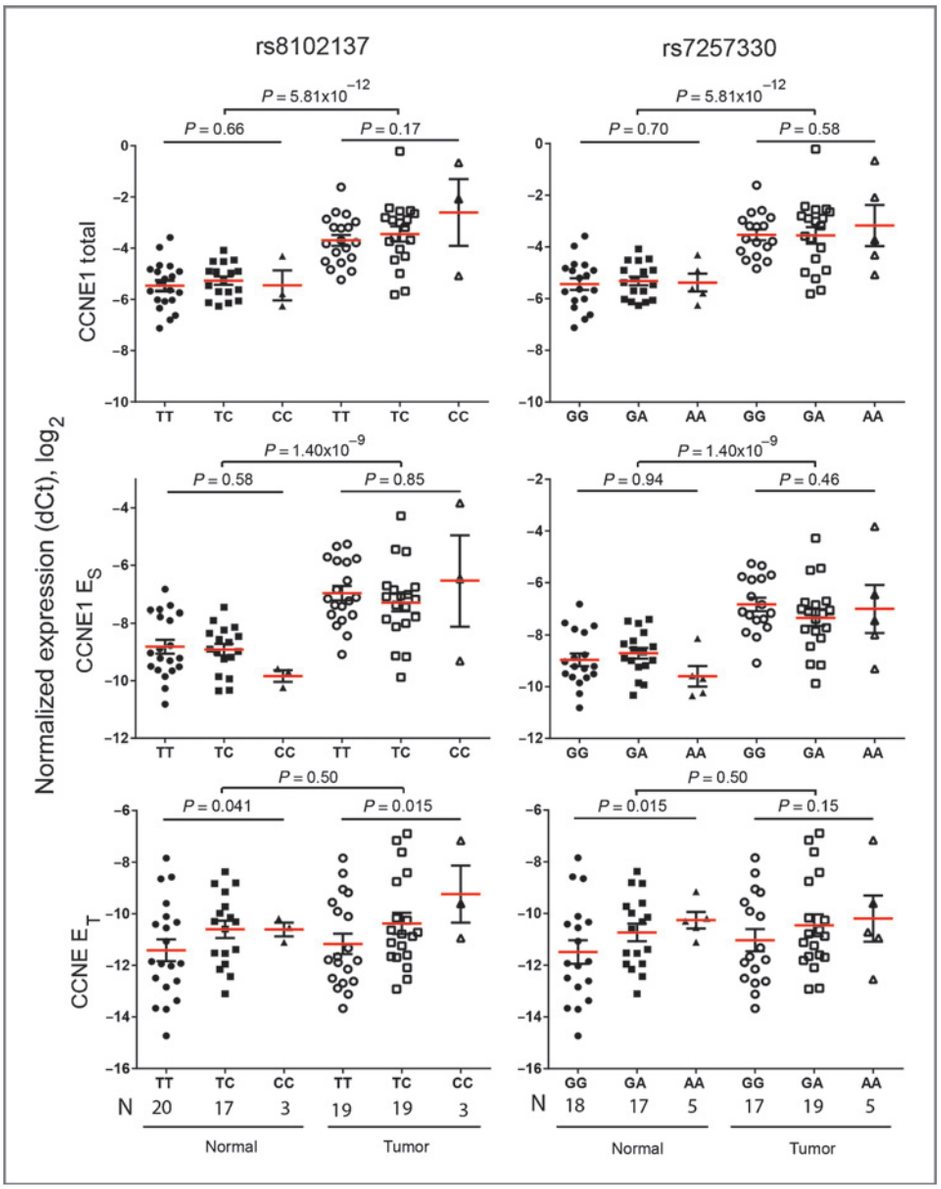

Figure 4. Analysis of *CCNE1* mRNA expression in bladder tumors and adjacent normal tissue and in relation to rs8102137 and rs7257330 genotypes. Expression of total *CCNE1* (all transcripts), *cyclin* E_S, and *cyclin* E_T transcripts was measured with TaqMan assays. *CCNE1* expression was normalized to a geometric mean of endogenous controls (*B2M*, *GAPDH,* and *PPIA*) and presented on the $\log_2$ 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.

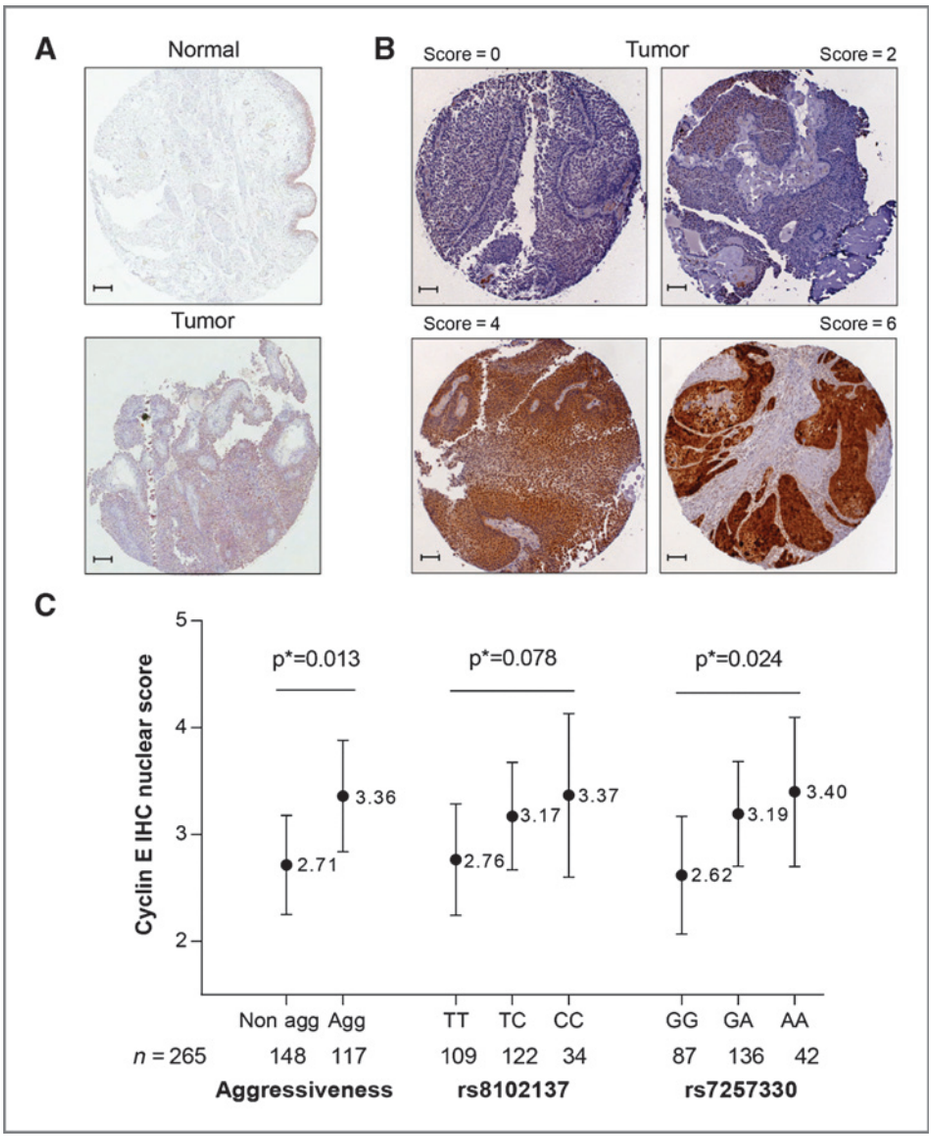

Figure 5. IHC analysis of cyclin E expression in bladder tissues. A, images of cyclin E expression in one pair of tumor-adjacent normal bladder tissue samples, representative of eight pairs tested. Immunostaining was done with an anti-CCNE1 rabbit polyclonal antibody (HPA018169; Sigma; dilution 1:85).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

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