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ORIGINAL REPORT

Prevalence and Co-Occurrence of Actionable Genomic Alterations in High-Grade Bladder Cancer

Gopa Iyer, Hikmat Al-Ahmadie, Nikolaus Schultz, Aphrothiti J. Hanrahan, Irina Ostrovnaya, Arjun V. Balar, Philip H. Kim, Oscar Lin, Nils Weinhold, Chris Sander, Emily C. Zabor, Manickam Janakiraman, Ilana R. Garcia-Grossman, Adriana Heguy, Agnes Viale, Bernard H. Bochner, Victor E. Reuter, Dean F. Bajorin, Matthew I. Milowsky, Barry S. Taylor, and David B. Solit

Author affiliations appear at the end of this article.

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Corresponding author: David B. Solit, MD, Human Oncology and Pathogenesis Program, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021; e-mail: solitd@mskcc.org.

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ABSTRACT

Purpose
We sought to define the prevalence and co-occurrence of actionable genomic alterations in patients with high-grade bladder cancer to serve as a platform for therapeutic drug discovery.

Patients and Methods
An integrative analysis of 97 high-grade bladder tumors was conducted to identify actionable drug targets, which are defined as genomic alterations that have been clinically validated in another cancer type (eg, *BRAF* mutation) or alterations for which a selective inhibitor of the target or pathway is under clinical investigation. DNA copy number alterations (CNAs) were defined by using array comparative genomic hybridization. Mutation profiling was performed by using both mass spectroscopy-based genotyping and Sanger sequencing.

Results
Sixty-one percent of tumors harbored potentially actionable genomic alterations. A core pathway analysis of the integrated data set revealed a nonoverlapping pattern of mutations in the RTK-RAS-RAF and phosphoinositide 3-kinase/AKT/mammalian target of rapamycin pathways and regulators of G_1-S cell cycle progression. Unsupervised clustering of CNAs defined two distinct classes of bladder tumors that differed in the degree of their CNA burden. Integration of mutation and copy number analyses revealed that mutations in *TP53* and *RB1* were significantly more common in tumors with a high CNA burden ($P < .001$ and $P < .003$, respectively).

Conclusion
High-grade bladder cancer possesses substantial genomic heterogeneity. The majority of tumors harbor potentially tractable genomic alterations that may predict for response to target-selective agents. Given the genomic diversity of bladder cancers, optimal development of target-specific agents will require pretreatment genomic characterization.

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INTRODUCTION

Approximately 70,000 cases of bladder cancer are diagnosed annually in the United States with more than 14,000 disease-related deaths.[1] In the metastatic setting, bladder cancer is almost always incurable and no systemic therapy has been shown to prolong survival in patients who have progressed following first-line cisplatin-based chemotherapy. Novel approaches are thus urgently needed for this common, highly lethal malignancy.

The realization that tumors are dependent on driver mutations that promote and maintain the malignant phenotype, even when in an advanced state, has led to dramatic improvements in anticancer therapy. Increasingly, cancers are classified by the ensemble of mutant genes they harbor; targeting such genes with selective inhibitors has resulted in profound clinical impact in lung adenocarcinomas with EGFR mutations or EML4-ALK rearrangements, BRAF-mutant melanomas, and in several additional genetically defined solid tumors.[2-5] The extent, duration, and rates of response for many of these examples are unprecedented and have led to rapid drug approval in molecularly defined patient subsets.

With the goal of accelerating the adoption of a precision medicine-based approach for patients with advanced bladder cancer, we performed an integrated genomic analysis to define the prevalence and patterns of co-occurrence of therapeutically tractable genomic alterations in 97 high-grade

urothelial tumors. We identified discrete subsets of bladder cancers defined by the presence of actionable alterations in the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways as well as regulators of G_1-S phase cell cycle progression. Our findings indicate that although bladder cancer, as defined by site of origin, is genetically heterogeneous, therapies targeting specific alterations may prove effective if patients with appropriate molecular profile are identified prospectively.

PATIENTS AND METHODS

Mutation Detection

All tumor samples and associated clinical data were collected retrospectively under a protocol approved by the institutional review board or waiver of authorization. Tumors were snap-frozen at resection. A reference cryostat section stained with hematoxylin and eosin was reviewed for every sample by a board-certified genitourinary pathologist (H.A.-A.), and samples were macrodissected to ensure ≥ 60% tumor content. Mutation analysis was performed by using both mass spectrometry–based genotyping and Sanger sequencing as previously described.[6,7]

DNA Copy Number Profiling

Labeled tumor DNA was cohybridized to Agilent 1 M human oligonucleotide comparative genomic hybridization microarrays with commercially available reference normal DNA (Roche, Basel, Switzerland). Raw copy number estimates were normalized, segmented by using circular binary segmentation, and analyzed by using the RAE algorithm as previously described.[8] Regions with false discovery rates less than 1% were considered significant; regions of statistically significant genomic alterations were excluded if they were known or presumed germline copy number polymorphisms or if they overlapped with previously identified variants. All genome coordinates were based on the National Center for Biotechnology Information build 36.1 (hg18) of the reference human genome.

RNA Expression Profiling

Total RNA was extracted by using the RNeasy kit (Qiagen, Germantown, MD), labeled (TotalPrep RNA Labeling Kit; Ambion, Carlsbad, CA), and hybridized to Human HT-12 Expression BeadChip arrays (Illumina, San Diego, CA). Arrays were scanned on a BeadArray Reader (Illumina), and gene-level messenger RNA data were normalized by using quantile normalization.[9] The E2F3 expression signature score was calculated by summing the expression scores for a panel of genes regulated by the E2F3 transcription factor.

Drug Sensitivity Testing

Cell lines underwent DNA fingerprinting to exclude the possibility of mislabeling or cross-contamination.[6] Cell viability was assayed via trypan blue exclusion. Immunoblot analysis was performed to assess the effect of MK2206 (2.5 μmol/L) treatment on downstream PI3K/AKT pathway intermediates.

Statistical Analysis

Overall survival (OS) was defined as time from date of surgery to death as a result of any cause, and time to recurrence was defined as time from surgery to recurrence with pathologic confirmation or, if not present, with Response Evaluation Criteria in Solid Tumors (RECIST)–based radiographic confirmation. Association of specific copy number alterations (CNAs) with time to recurrence and OS was tested by using the log-rank test and univariate Cox proportional hazards regression. OS between tumor cohorts was compared by using stratified Cox regression. Cancer-specific survival was compared by using a competing risk model. For disease-specific survival analysis, deaths from other and unknown causes were combined and treated as competing events. Competing risk analysis was performed using the "cmprsk" R package. Associations between binary variables (eg CNA status [high *v* low], stage [I to II *v* III to IV]), treatment with neoadjuvant chemotherapy, and point mutations and CNAs were tested by using Fisher's exact test. Wilcoxon rank sum testing was used to compare the total number of alterations with clinical parameters.

Table 1. Select Clinical Characteristics of 97 High-Grade Urothelial Carcinoma Samples

Clinical Characteristic	No.	%
Age, years		
Median	73	
Range	42-89	
Sex		
Male	72	74
Female	25	26
Specimen		
Cystectomy	94	97
Nephroureterectomy	1	1
TURBT	2	2
Surgical stage (95 tumors)		
0 (pTa or pTis)	4	4
I	11	11
II	15	16
III	33	35
IV	32	34
Neoadjuvant chemotherapy	34	35

Abbreviation: TURBT, transurethral resection of bladder.

Data Availability

Data are publically available through the Memorial Sloan-Kettering Cancer Center (MSKCC) cBioPortal for Cancer Genomics.[10,10a]

RESULTS

We performed an integrated analysis of 97 high-grade urothelial tumors (Table 1). Patients with these tumors carry a substantial risk for recurrence and subsequent disease-specific mortality. Fifty-seven tumors (59%) were histopathologically classified as transitional cell carcinoma not otherwise specified. Thirty patients (31%) had predominantly transitional cell carcinoma with additional minor histologic components (Data Supplement). Ten samples (10%) displayed predominantly neuroendocrine features.

To confirm that this cohort was representative of patients with bladder cancer who were treated at our institution, we identified a contemporary comparator cohort of 285 consecutively treated age-, sex-, and stage-matched patients (in a 3:1 ratio). Patients in this comparator cohort had undergone radical cystectomy for bladder cancer at MSKCC between 2007 and 2010. The frequency of histologic subtypes between cohorts was comparable with the exception of neuroendocrine tumors, which were not present in the comparator cohort. The median OS times for the comparator and study cohorts were 38 and 35.2 months, respectively ($P = .19$; Data Supplement), with similar cancer-specific survivals ($P = .61$).

To define the landscape of CNAs in high-grade bladder cancers, we performed high-resolution array comparative genomic hybridization. Unsupervised clustering of segmented copy number events revealed two distinct tumor subsets differing in their degree of CNA burden (high-burden *v* low-burden CNA; Fig 1A). Tumor content, as estimated by pathologic review, was similar between the low-burden and high-burden CNA clusters, indicating that differences in the extent of copy number events between clusters could not be attributed to

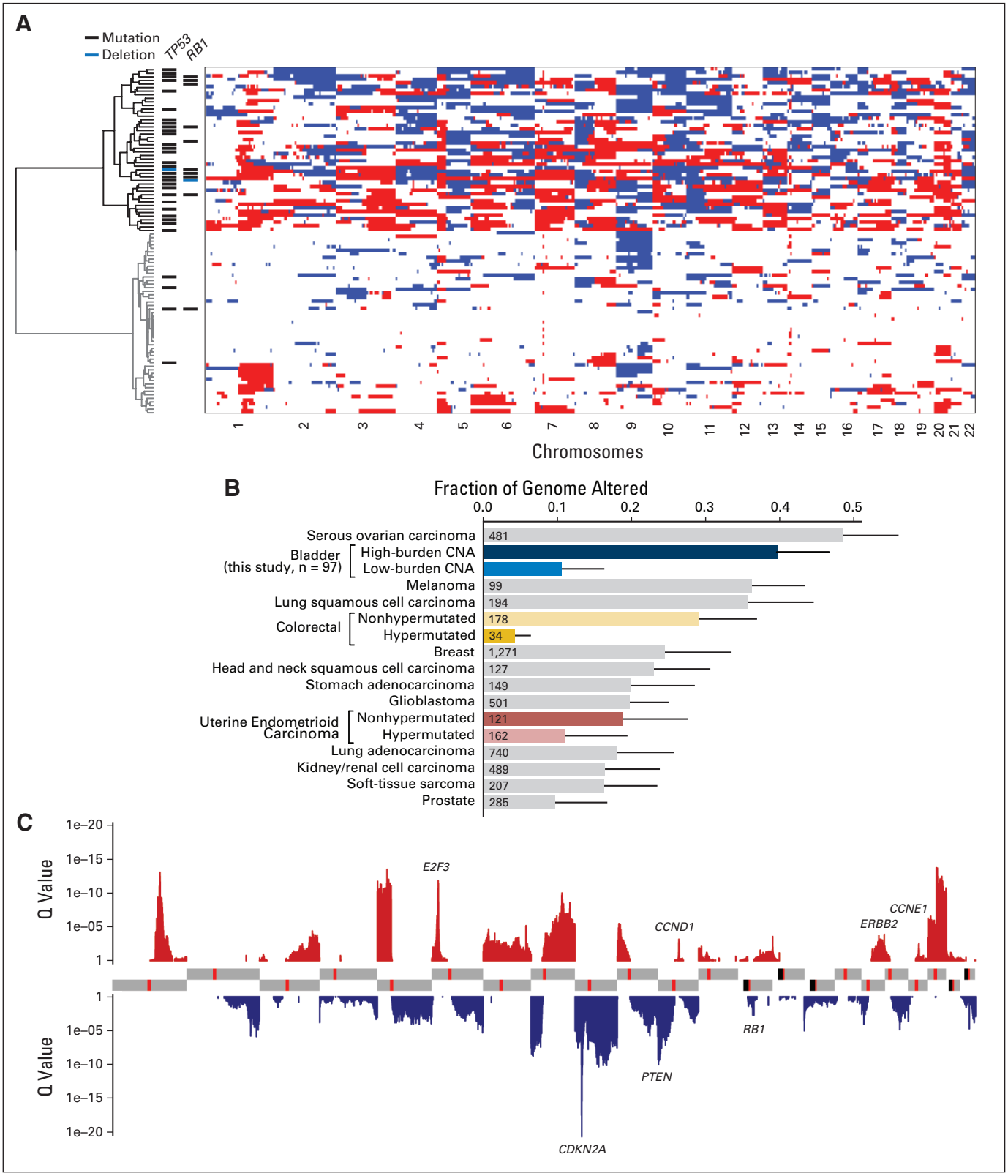

Fig 1. Landscape of DNA copy number alterations (CNAs) in high-grade bladder cancer. (A) Unsupervised hierarchical clustering of array comparative genomic hybridization data identified two distinct classes of bladder cancers. *TP53* and *RB1* alterations were significantly more common in the high copy number aberrant subset. (B) Fraction of the genome altered in the subsets of bladder tumors with high and low copy number aberrations and in additional select human cancers. The cohort of copy number data used for comparison was derived from multiple large tumor collections analyzed by array comparative genomic hybridization (both published and unpublished data) and includes The Cancer Genome Atlas tumor types with more than 100 available samples. It represents a composite, unbiased data set for comparing global copy number changes across myriad tumor subtypes (see Data Supplement for list of references from which these data were derived). (C) Statistically significant genomic amplifications (red) and deletions (blue) inferred from RAE analysis are indicated across the autosomes. Select genes located within recurrently amplified or deleted regions are highlighted.

differences in stromal contamination. We compared the CNA burden of the study cohort to 5,135 tumors from 14 diverse nonhematologic tumor types (Fig 1B). Only serous ovarian cancers demonstrated a greater degree of mean structural aberration than the high-burden CNA bladder tumors. In contrast, the degree of structural aberration in the low-burden CNA bladder cancers was similar to hypermutated uterine endometrioid and prostate cancers and greater only than hypermutated colorectal cancers. Although two subsets of genetically distinct tumors could be defined by CNA burden, these did not define clinically distinct disease subsets. Specifically, there was no significant difference in the rate of recurrence-free, OS, or cancer-specific survival between the two groups ($P = .98$, $P = .75$, and $P = .49$, respectively), with both demonstrating a high bladder cancer–specific mortality (Data Supplement).

To identify recurrent, functionally significant CNAs that may represent driver events, we used the statistical method RAE (Fig 1C; Data Supplement). Notable regions of amplification included 11q13.2 to 13.3 spanning the *CCND1* gene and the 17q12 locus encoding the *ERBB2* gene. The most commonly deleted locus was 9p21.3 spanning the *CDKN2A* and *CDKN2B* tumor suppressor genes. The most frequent arm-length gains and losses were 20q (41.2%) and 11p (36.1%), respectively. These results are consistent with the results of previous array-based studies that identified partial or complete loss of chromosome 9 as well as 20q and 17q21 gain as common events in bladder cancer.[11-13]

Because alterations in gene copy number represent only one mechanism of oncoprotein dysregulation, we performed mutation profiling of all 97 tumors. Using a mass spectrometry–based Sequenom iPLEX assay, we detected hotspot alterations in select cancer genes.[6,7] Sanger sequencing of all coding exons of 15 oncogenes and tumor suppressor genes, selected for their potential as predictive biomarkers of response to targeted agents, was also performed (Data Supplement). Sixty-three samples (65%) harbored mutations in at least one gene, the most prevalent of which were *TP53* (34%), *FGFR3* (13%), and *PIK3CA* (18%). Integration of mutation and copy number data revealed that *TP53* and *RB1* alterations were significantly more common in high-burden CNA tumors ($P < .001$ and $P < .003$, respectively; Fig 1A). Correlations between survival, stage, neoadjuvant chemotherapy, and select aberrations are described in the Data Supplement.

To better define the co-occurrence pattern of mutations and CNAs, we grouped genes into core signal transduction pathways or canonical cell functions. MAPK pathway alterations were identified in 35% of samples (Fig 2A). The most commonly altered genes were *FGFR1*, *FGFR3*, *ERBB2*, *MET*, *NF1*, *KRAS*, and *BRAF*, each occurring in 2% to 13% of patients and arising in a predominantly mutually exclusive pattern, suggesting that these events confer overlapping phenotypic effects. Because *ERBB2* amplification is a validated drug target in breast and esophagastric tumors,[14-16] we further explored the significance of the *ERBB2* amplifications (six patients [6.2%]) in our tumor cohort. Focal *ERBB2*-amplified tumors were found to exhibit increased ERBB2 messenger RNA expression as compared with nonamplified samples and 3+ HER2 protein overexpression by immunohistochemistry (Figs 2B and 2C).

Consistent with studies of other solid tumors,[17,18] *TP53* mutation and *MDM2* (which encodes an E3 ubiquitin ligase for p53) amplification occurred in a nonoverlapping distribution (Fig 3A). Alteration in genes that regulate G_1-S phase transition were also highly prevalent (60% of all tumors analyzed; Fig 3B). Specifically, mutually exclusive focal amplifications of *CCND1* and *CCNE1* were present in 14% and 5% of samples, respectively, and were inversely correlated with the presence of *RB1* mutations or deletions. A similar pattern of mutual exclusivity was not observed among these three genes and *E2F3* amplification (21%) or *CDKN2A* deletion or mutation (24%).

Amplification of the *E2F3* locus was notable, because amplification of this region rarely occurs in other epithelial tumor types (21% *v* 4.9% of 1,932 nonurothelial epithelial tumors) and was associated with more advanced stage (28% stage III to IV *v* 6.7% stage I to II; $P = .028$).[19] Thus, *E2F3* amplification may represent a lineage-specific event in bladder cancer, similar to *MITF* amplification in metastatic melanoma. To assess the functional impact of *E2F3* amplifications in bladder cancer, we calculated an E2F3 activity score from the expression levels of 23 experimentally validated E2F3 target genes[20] (Data Supplement). Tumor samples with *E2F3* amplifications or *RB1* mutations or homozygous deletions exhibited high E2F3 signature scores (Fig 3B). Samples with *CCND1* or *CCNE1* amplification had, on average, lower E2F3 signature scores, whereas tumors with either *CDKN2A* alterations or those lacking alterations in all five genes exhibited the lowest scores. Nevertheless, the latter group of wild-type tumors expressed the E2F3 signature heterogeneously, suggesting the presence of occult alterations that phenocopy the effects of *E2F3* amplification and/or *RB1* inactivation in some *E2F3/RB1*–wild-type bladder tumors. Expression profiling data indicated a strong concordance between copy number aberration and expression score for all genes except *RB1* (Data Supplement).

Because *RB1* alterations are found in the majority of neuroendocrine lung cancers,[21] we correlated *RB1* and *E2F3* alterations with the presence of neuroendocrine histology. Both *E2F3* amplification and *RB1* deletion/mutation were more prevalent in the subset of tumors exhibiting neuroendocrine differentiation compared with those with predominantly urothelial morphology (*E2F3*, 50% *v* 17%; $P = .03$; *RB1*, 50% *v* 13%; $P = .01$). Tumors containing neuroendocrine morphology also had higher rates of *TP53* alterations and a higher CNA burden (Data Supplement).

Collectively, our integrated analysis revealed that although bladder cancers are genetically heterogeneous, subsets of tumors could be identified by discrete, nonoverlapping alterations that could serve as the basis for therapeutic intervention. Notably, 61% of tumors harbored an actionable alteration (events that, on the basis of clinical or preclinical data, would be predicted to confer sensitivity to selective inhibitors approved for use in other malignancies or currently in clinical testing). To determine whether the genomic heterogeneity of the human disease could be modeled in preclinical studies, we performed a similar integrative analysis on a panel of bladder cancer cell lines. We found only a modest overlap between the genomic profile of the tumor and cell line cohorts. For example, none of the bladder cancer cell lines had focal amplification of the *ERBB2* gene. Nevertheless, several potentially actionable alterations were observed in both the tumor and cell line panels. In particular, aberrations in genes within the PI3K/AKT signaling pathway were highly prevalent in both (Fig 4A; Data Supplement).

To explore the functional significance of PI3K/AKT alterations in bladder cancer, we assessed the AKT dependence of a subset of the bladder cancer cell lines as a function of their pathway mutation status by using MK2206, a highly selective, allosteric inhibitor of AKT1, AKT2, and AKT3. We chose this drug for its therapeutic relevance,

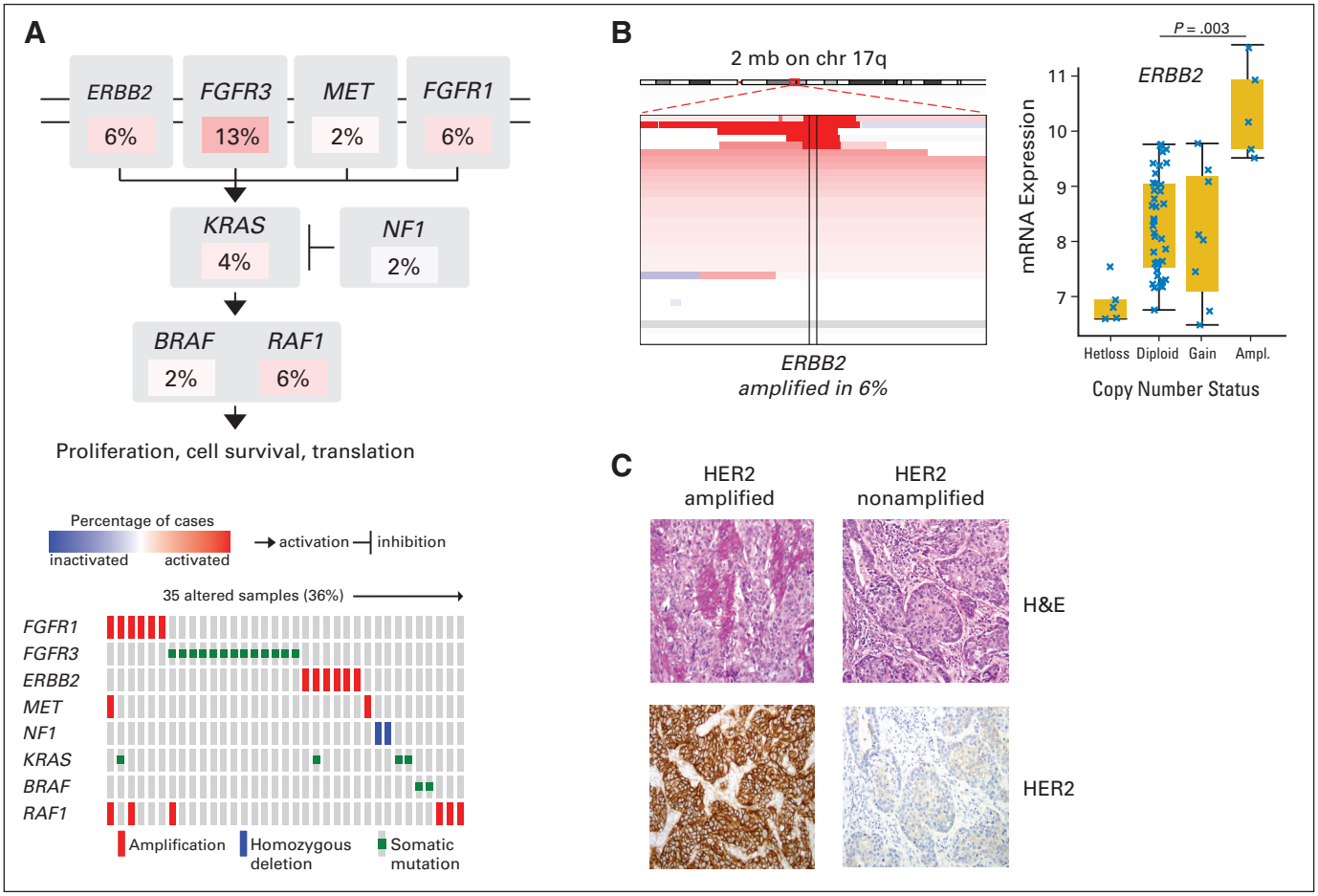

Fig 2. Co-occurrence of alterations within the RTK/RAS/RAF signaling pathway in high-grade bladder cancer. (A) Incidence (%) of amplifications, deletions, and mutations of select receptor tyrosine kinases and downstream targets. The heatmap compares the distribution of each alteration across tumor samples (bottom). (B) *ERBB2*-amplified samples are highlighted (left), and *ERBB2* transcript expression levels as a function of *ERBB2* gene copy number are indicated (right). *ERBB2*-amplified samples also exhibited significantly increased messenger RNA (mRNA) expression levels as compared with diploid samples. (C) Immunohistochemistry analysis of a representative *ERBB2*-amplified tumor sample exhibiting 3+ human epidermal growth factor receptor 2 (HER2) overexpression and an *ERBB2*-nonamplified tumor sample shown for comparison (right). Ampl., amplified; H&E, hematoxylin and eosin; Hetloss, heterozygous loss.

since it is currently being investigated in multiple solid tumor malignancies. Cell lines harboring *PIK3CA* and *AKT1* mutations were highly sensitive to MK2206, those with alterations in upstream pathway components including FGFR-3 and HRAS less so, whereas cell lines with downstream TSC-1 alterations were resistant to AKT inhibition (Fig 4B). In bladder cancer cells with *PIK3CA* mutations, treatment with MK2206 resulted in downregulation of phosphorylated AKT and inhibition of downstream AKT effectors including FOX-01/03, PRAS40, S6, and 4EBP (Fig 4C). Treatment of TSC-1–null bladder cancer cells with MK2206 similarly resulted in potent inhibition of AKT activation and proximal AKT effectors, including phospho–FOX-01/03 and phospho-PRAS40, but in contrast to the effects of AKT inhibition in *PIK3CA*-mutant cells, a significant reduction in phosphorylated S6 and 4EBP1 expression was not observed. Therefore, although PI3K/AKT/mammalian target of rapamycin (mTOR) pathway mutations are common in bladder cancer, the sensitivity of bladder tumors to selective inhibitors of this pathway will be dictated by the underlying genetic determinant of pathway activation.

DISCUSSION

This integrative genomic analysis was performed to define the prevalence and co-occurrence of actionable drug targets in patients with high-grade bladder cancer. Sixty-one percent of tumors harbored potentially actionable alterations, including clinically validated drug targets such as focal, high-level *ERBB2* amplification[16] and *BRAF* and *TSC1* mutations.[5,22,23] A large proportion of tumors also harbored mutations and/or gene amplifications for which targeted inhibitors are currently in advanced clinical testing, including *FGFR3* and *PIK3CA* mutations, *PTEN* deletions, and *FGFR1*, *CCND1*, and *MDM2* amplifications.[7,24-26] The largely nonoverlapping distribution of abnormalities in potentially actionable genes revealed by our core pathway analysis suggests that these alterations likely represent driver events and should be evaluated as drug targets in this disease. The Data Supplement lists select alterations found in our study cohort for which targeted therapies are currently available or under investigation.

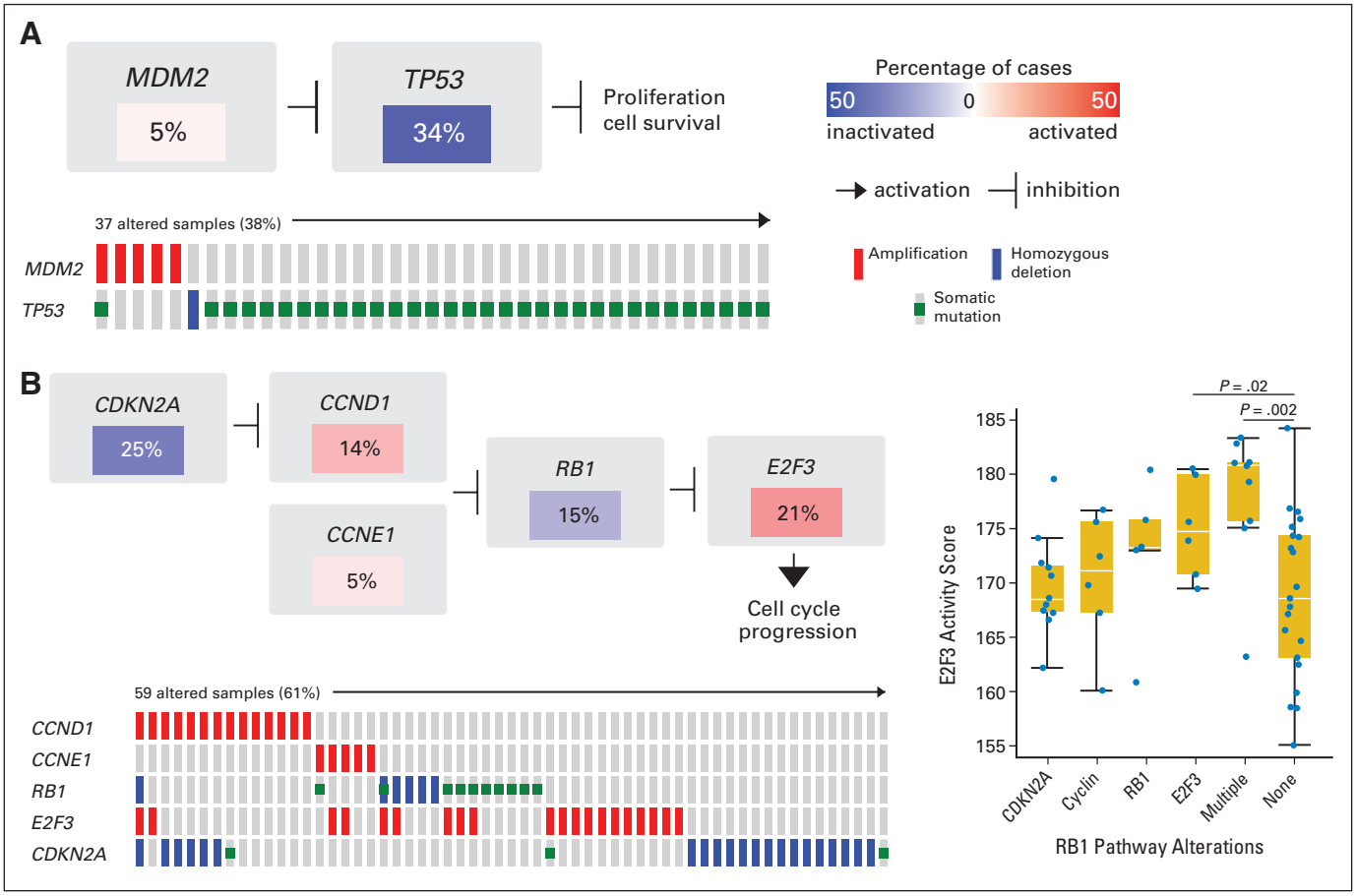

Fig 3. Alterations within the TP53 and RB1/E2F3 pathways in high-grade bladder cancer. (A) Heatmap showing the distribution of *TP53* and *MDM2* alterations. (B) The RB1 cell cycle regulatory pathway with incidence of mutations and copy number alterations displayed for each gene along with a heatmap showing the co-occurrence of genetic alterations in this pathway. The boxplot (right) depicts the association between an E2F3 expression signature score and alterations within genes involved in the RB1 pathway ("multiple" refers to more than one gene in this pathway altered in the same tumor sample). Those samples harboring *E2F3* amplification or amplification of multiple genes within the RB1 pathway display significantly increased E2F3 activity scores compared with tumors lacking amplification of any component of the pathway (labeled as "none"). *P* values were calculated by *t* test.

Our integrated analysis revealed that bladder cancer is a genetically heterogeneous disease with no single pathognomonic molecular event. Tumors could be divided into two discrete subsets (high- or low-burden CNAs) and the degree of CNA correlated with the presence of *TP53* and/or *RB1* abnormalities. This result is consistent with prior studies showing that TP53 and RB1 inactivation induces genomic instability and promotes aneuploidy.[27,28] Although not clinically distinct, the strong correlation between the degree of structural aberration and *TP53* and *RB1* mutation status suggests that these events play a central role in the pathogenesis of high-burden CNA bladder cancers.

Because functional studies using human cancer cell lines have historically served as the basis for the clinical development of novel cancer therapies, we compared the genomic profile of the high-grade bladder cancers to a collection of bladder cancer cell lines commonly used in preclinical studies. We found only a modest overlap between the genomic profiles of the tumors and cell lines with some potential drug targets, such as *ERBB2* amplification, not represented in the cell lines. These results indicate that a significantly larger panel of bladder cancer cell lines will be needed to model the genomic complexity of this disease. Because genomic alterations in the PI3K/AKT/mTOR pathway were common in both, a subset of cell lines was used to determine whether mutation status predicted for sensitivity to the selective AKT inhibitor MK2206. Consistent with data from breast and ovarian cancers,[24,29] treatment of *PIK3CA*- and *PTEN*-mutant cell lines with MK2206 was associated with modulation of AKT, its downstream effectors, and cell proliferation. In contrast, cell lines with mutations in TSC-1, a downstream effector of the PI3K/AKT pathway, were resistant to MK2206 despite potent inhibition of AKT and the proximal AKT substrates PRAS40 and FOX-01/03. AKT effectors downstream of TSC1, including S6 and 4EBP1, were not inhibited by MK2206 in TSC1 null cells, a result analogous to the resistance of KRAS-mutant cells to EGFR-directed therapies.[30,31] These results suggest that response to selective inhibitors of kinase signaling pathways in bladder cancer may vary as a function of the molecular basis of pathway activation.

There were several limitations inherent to the sample set analyzed in this study. The inclusion of tumors with predominantly neuroendocrine histology in the study cohort may have influenced the prevalence of some genetic aberrations: neuroendocrine tumors were more likely to harbor alterations in *TP53*, *RB1*, and *E2F3*. Furthermore, it is possible that the prevalence of actionable alterations may differ

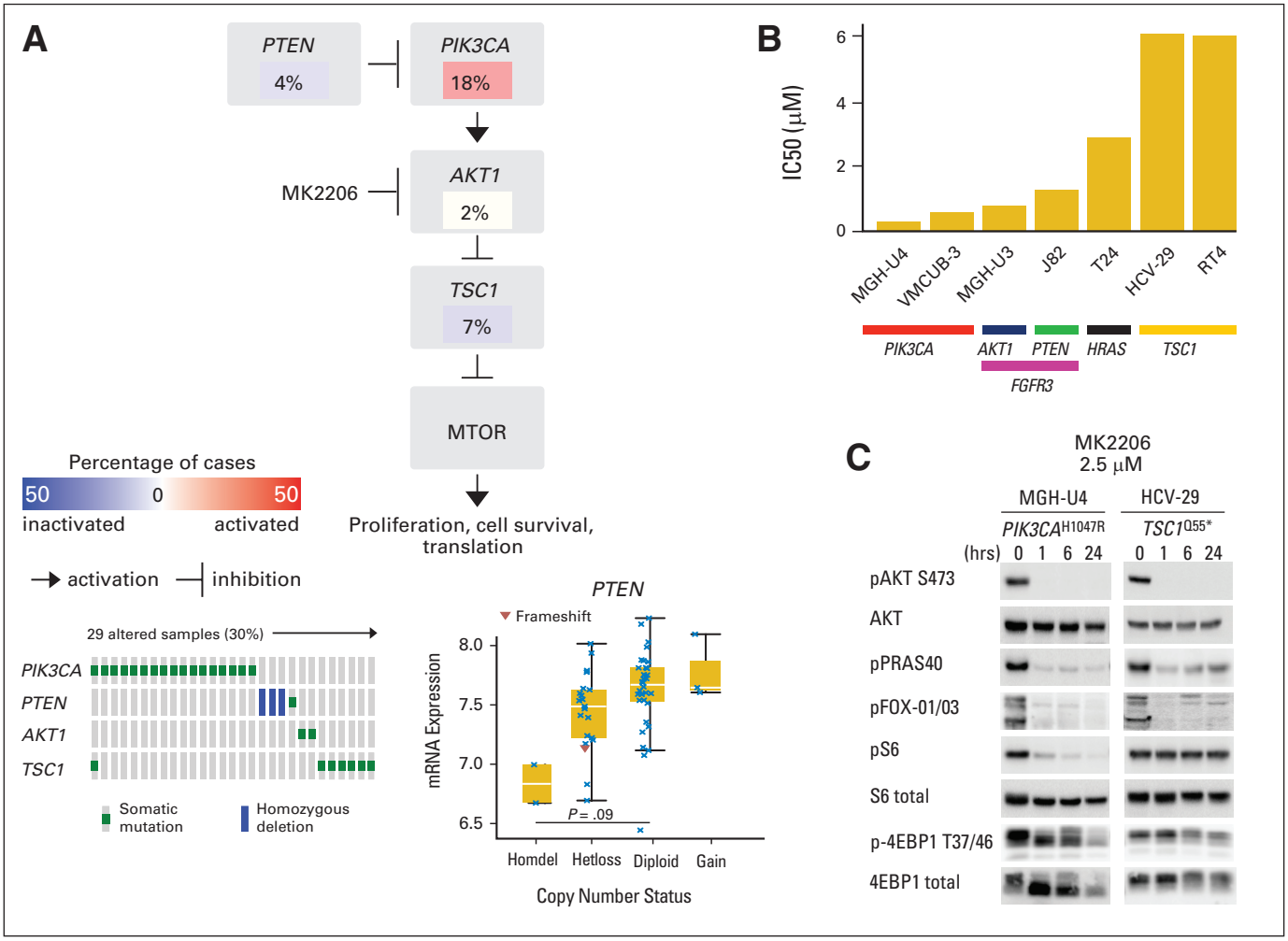

Fig 4. Alterations within the phosphoinositide 3-kinase (PI3K)/AKT pathway in high-grade bladder cancer. (A) Key components of the PI3K/AKT signaling pathway displayed with their incidence of mutations and copy number abnormalities. The corresponding heatmap shows the distribution of pathway alterations across the tumor cohort. The mRNA expression score for *PTEN*-deleted samples is shown to the right of the heatmap. *P* value comparing expression scores between samples harboring homozygous deletion (homdel) versus diploid samples calculated by *t* test. (B) IC50 (concentration that inhibits 50%) values for a panel of urothelial cell lines with distinct PI3K/AKT pathway alterations (as annotated) as well as one cell line with an *HRAS* mutation. Each cell line was exposed to increasing concentrations of MK2206 and harvested 5 days after addition of drug. Cell viability was measured using trypan blue exclusion. (C) Immunoblot analysis of PI3K/AKT pathway downstream targets after exposure to MK2206 in the MGH-U4 (*PIK3CA* H1047R) and the HCV-29 (*TSC1* Q55*) cell lines. Cell were harvested at 0, 1, 6, and 24 hours after addition of drug. Hetloss, heterozygous loss.

between primary and metastatic tumors. An analysis of matched primary and metastatic samples from the same patient would address this possibility and should be a focus of future research efforts. Finally, several reports in other tumor types have illustrated the potential impact of intratumoral heterogeneity on the efficacy of targeted agents.[32] Approximately one third of the tumors in this study exhibited minor histologic components such as squamous or sarcomatoid differentiation, a result similar to a historical series at our institution in which 27% of tumors evinced divergent differentiation (unpublished data). Emerging next-generation methodologies may allow for a determination of whether such intratumoral histologic heterogeneity reflects intratumoral genomic heterogeneity and/or polyclonality.

In conclusion, we find that approximately 60% of bladder cancers harbor potentially actionable genetic alterations that have either been validated as drug targets in other solid tumors or for which selective inhibitors are currently in early clinical testing. Bladder cancers, however, exhibit significant genetic heterogeneity, with most potentially actionable alterations identified in a minority of patients. Therefore, an effective drug for which activity is restricted to a molecularly defined bladder cancer subtype would likely be deemed clinically inactive if tested in a nonenriched patient population. Our results suggest that the failure of target-directed therapies in bladder cancer to date is not necessarily a function of the lack of tractable drug targets but rather the genomic heterogeneity of the disease. Until recently, prospective characterization of individual patients was not feasible because of technical and cost limitations. The findings presented here underscore the need for prospective genomic characterization of patients with bladder cancer to enrich future clinical trials with patients whose tumors harbor alterations in the drug target of interest.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Gopa Iyer, Hikmat Al-Ahmadie, Aphrothiti J. Hanrahan, Bernard H. Bochner, Dean F. Bajorin, Matthew I. Milowsky, David B. Solit
Collection and assembly of data: Gopa Iyer, Hikmat Al-Ahmadie, Aphrothiti J. Hanrahan, Arjun V. Balar, Philip H. Kim, Oscar Lin, Manickam Janakiraman, Ilana R. Garcia-Grossman, Adriana Heguy, Agnes Viale, Barry S. Taylor, David B. Solit
Data analysis and interpretation: Gopa Iyer, Hikmat Al-Ahmadie, Nikolaus Schultz, Aphrothiti J. Hanrahan, Irina Ostrovnaya, Nils Weinhold, Chris Sander, Emily C. Zabor, Manickam Janakiraman, Victor E. Reuter, Dean F. Bajorin, Matthew I. Milowsky, Barry S. Taylor, David B. Solit
Manuscript writing: All authors
Final approval of manuscript: All authors

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Affiliations

Gopa Iyer, Hikmat Al-Ahmadie, Nikolaus Schultz, Aphrothiti J. Hanrahan, Irina Ostrovnaya, Arjun V. Balar, Philip H. Kim, Oscar Lin, Nils Weinhold, Chris Sander, Emily C. Zabor, Manickam Janakiraman, Ilana R. Garcia-Grossman, Adriana Heguy, Agnes Viale, Bernard H. Bochner, Victor E. Reuter, Dean F. Bajorin, and David B. Solit, Memorial Sloan-Kettering Cancer Center; Victor E. Reuter, Dean F. Bajorin, and David B. Solit, Weill Medical College, Cornell University, New York, NY; Matthew I. Milowsky, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC; and Barry S. Taylor, Helen Diller Family Comprehensive Cancer Center, University of California at San Francisco, San Francisco, CA.

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