Biology of Human Tumors

Concurrent Alterations in TERT, KDM6A, and the BRCA Pathway in Bladder Cancer

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Abstract

Purpose: Genetic analysis of bladder cancer has revealed a number of frequently altered genes, including frequent alterations of the telomerase (TERT) gene promoter, although few altered genes have been functionally evaluated. Our objective is to characterize alterations observed by exome sequencing and sequencing of the TERT promoter, and to examine the functional relevance of histone lysine (K)-specific demethylase 6A (KDM6A/UTX), a frequently mutated histone demethylase, in bladder cancer.

Experimental Design: We analyzed bladder cancer samples from 54 U.S. patients by exome and targeted sequencing and confirmed somatic variants using normal tissue from the same patient. We examined the biologic function of KDM6A using in vivo and in vitro assays.

Results: We observed frequent somatic alterations in BRCA1 associated protein-1 (BAP1) in 15% of tumors, including deleterious alterations to the deubiquitinase active site and the nuclear localization signal. BAP1 mutations contribute to a high frequency of tumors with breast cancer (BRCA) DNA repair pathway alterations and were significantly associated with papillary histologic features in tumors. BAP1 and KDM6A mutations significantly co-occurred in tumors. Somatic variants altering the TERT promoter were found in 69% of tumors but were not correlated with alterations in other bladder cancer genes. We examined the function of KDM6A, altered in 24% of tumors, and show depletion in human bladder cancer cells, enhanced in vitro proliferation, in vivo tumor growth, and cell migration.

Conclusions: This study is the first to identify frequent BAP1 and BRCA pathway alterations in bladder cancer, show TERT promoter alterations are independent of other bladder cancer gene alterations, and show KDM6A loss is a driver of the bladder cancer phenotype. Clin Cancer Res; 20(18); 4935–48. ©2014 AACR.

Introduction

Bladder cancer is the fifth most common cancer worldwide (1), with 386,300 new cases and 150,200 deaths in 2008 (1). Bladder cancer is classified into 2 types that are thought to be driven by mutations in different sets of genes (2). The low-grade and nonmuscle invasive (NMI) form is successfully treated with transurethral surgery and intravesical immunotherapy (2) and accounts for ~80% of all cases. Alterations associated with this form include mutation or overexpression of HRAS, fibroblast growth factor receptor 3 (FGFR3), and the KDM6A; refs. 2–4. The remaining 20% of cases are muscle invasive (MI) and, despite aggressive treatment with cystectomy, have a 5-year survival rate of <50% (2). Overexpression or mutation of human epidermal growth factor receptor-2 (HER2), epithelial growth factor receptor (EGFR), TP53, and RBP1 are associated with MI bladder cancer (2–4).

Next-generation sequencing (NGS) of the exomes of 9 bladder cancer tumors revealed frequent somatic alterations in 54 genes, including 16 new bladder cancer genes mutated in ≥5% of cases (3). Eight were genes encoding chromatin modifying/remodeling enzymes, AT rich interactive domain 1A (ARID1A), CHD6, CREBBP, EP300, MLL, MLL3, NCOR1, and KDM6A. Recently, analysis of 99 bladder cancer tumors by whole genome, whole exome, and transcriptome NGS (5) revealed additional altered genes,
including stromal antigen 2 (STAG2) and ESPL1, encoding proteins involved in the spindle checkpoint of the cell cycle, and a recurrent fusion involving FGFR3 and TACC3, another spindle checkpoint gene (6).

All tumors analyzed to date by NGS were from Chinese patients. Thus, findings may reflect the regional ethnicity of the patients, differences in exposure to damaging agents, or lifestyle factors. Here we determine by exome sequencing of 14 tumors if novel alterations could be found in a cohort of non-Asian patients diagnosed with bladder cancer in the United States. Given the recent report of TERT gene promoter mutations in bladder cancer (7), we examined the TERT promoter using targeted sequencing in the 14 tumors and in an additional 40 tumors. Healthy, noncancerous tissue DNA was available to determine variants that were tumor specific. Finally, given the frequent alteration of KDM6A found by this study and in Chinese patient bladder cancer tumors, we examined whether KDM6A expression has functional in vitro, in vivo, and clinical prognostic impact.

Materials and Methods

Human subjects, tumor and normal tissue samples, and cell lines

The study was approved by the University of Colorado Denver (UCD), Institutional Review Board (Colorado Multiple Institutional Review Board, CB F490, protocols 10-1365 and 09-913). Clinical details on patients and tumors used for exome and targeted sequencing are summarized in Supplementary Table S1. Tissue samples were snap frozen in liquid nitrogen and stored at −80°C until DNA was isolated using proteinase K digestion followed by phenol/chloroform extraction. Hematoxylin and eosin–stained (H&E) sections of tumors were prepared by standard methods and evaluated by a board certified genitourinary pathologist (S.M. Lucia). Only tumor samples with ≥85% purity were processed for DNA. DNA was isolated from normal adjacent bladder tissue that was manually reviewed by a pathologist (S.M. Lucia) to confirm samples were free of urothelial carcinoma in situ and low-grade urothelial dysplasia. Functional experiments utilized T24T and MGHU3, 2 human bladder cancer cell lines that have been used extensively were selected. PCR and Sanger sequencing of T24T DNA revealed a KDM6A homozygous c.G2683T (p.E895X, NM_021140) introducing a stop codon. This truncates 506 C-terminus amino acid (aa) (36% of 1401 AA total), including the catalytic jumonji C domain (aa 1099–1241). Because T24T was derived from a female patient, the wild-type (WT) copy of KDM6A was also lost. The KDM6A protein coding sequences and splice junctions (SI) were WT in MGHU3. T24T cells were cultured in Dulbecco’s Modified Eagle Medium/F12 with 2.5 mmol/L l-glutamine solution adjusted with 2.4 g/L sodium bicarbonate and 5% fetal bovine serum. MGHU3 were cultured in minimal essential medium with 2 mmol/L l-glutamine, Earle’s balanced salt solution (2.2 g/L sodium bicarbonate), and 10% fetal bovine serum. Cell lines have been tested and authenticated by single nucleotide polymorphism analysis.

Exome capture, sequencing, and analysis

Bladder tumor genomic DNA (3 μg, quantitated by fluorometer and agarose gel) was fragmented and the exome captured using probes for ~180,000 protein-coding exons and microRNA loci based on curated genes in the consensus coding sequence database (SureSelect Human All Exon Kit; Agilent). Libraries were sequenced on a HiSeq 2000 platform (Illumina) and base calls on paired-end, 100 bp reads were generated using the Genome Analyzer Pipeline, v. 1.3, and standard parameters. Additional details are available in the Supplementary Methods.

Variant validation

Selected variants identified in tumor exomes were examined in DNA from the tumor and normal adjacent bladder tissue. Primers were designed using Primer 3 and ExonPrimer (UCSC Genome Browser) as described in the Supplementary Methods. PCR utilized FastStart reagents (Roche) and GeneAmp 9700 thermal cyclers (ABI) at a 58°C annealing temperature for 30 cycles preceded by a 10 cycle, 5°C touchdown. PCR products were evaluated by 2% agarose gel electrophoresis and sequenced using Big Dye v3.1 reagents (ABI) and a 3730 Genetic Analyzer (ABI). Mutation Surveyor (Softgenetics) and Sequencher, v.4.8 (GeneCodes) were used for sequence analysis.

Copy number assessment

Copy number variation (CNV) was assessed using the relative signal intensity (RSI) of somatic and heterozygous germline variants in Sanger chromatograms (8, 9), which is an estimate of mutant/WT nucleotide peak heights in sequencing chromatograms. RSI are semiquantitative measures of relative allelic fractions in heterogeneous cancer samples comparable to allele frequencies obtained after subcloning. High RSI scores indicate allelic imbalance because of somatic loss of WT alleles or amplification of oncogenic mutant alleles in cancer samples.

Annotation of CRM-DA proteins and BAP1

Chromatin remodeling-DNA associated (CRM-DA) proteins were annotated using UniProtKB/Swiss-Prot, GeneCards, PhosphoSitePlus, Modbase, and The Protein Model Portal databases for selected isoforms: 729 aa BAP1 adapted from ref. 10; ARID1A, 2285 aa; KDM6A, 1401 aa; and STAG2, 1231 aa. Phosphorylation sites are recurrent observations by mass spectrometry (PhosphoSitePlus). The BAP1 ribbon

Translational Relevance

Sequencing of bladder cancers reveals hitherto unappreciated mutations in BAP1 and BRCA pathway genes, while finding that somatic TERT promoter alterations were independent of somatic alterations in other genes. These data suggest novel strategies with poly (ADP-ribose) polymerase inhibitors combined with DNA damaging agents may be effective in specific patients.
Gene expression datasets and network analysis

Gene expression datasets are described in Supplementary Information. The genes with somatic mutations confirmed by Sanger sequencing in a recent work (5) and the current manuscript were imported into Ingenuity Pathway Analysis (IPA; Ingenuity Systems) for bioinformatic analysis. IPA was used to group these mutated genes into gene-limited networks (35 genes maximum) based on evidence of direct or indirect relationships between genes according to the IPA Knowledge Base. The IPA network algorithm seeks to maximize the interconnectivity within a group of selected genes and scores networks based on a right tailed Fisher’s exact test that calculates the probability that the given relationships can be explained by a random model. The networks do not include all possible relationships for each member, because of size constraints placed on the network, and specific genes may appear in multiple networks. We identified differentially expressed genes in bladder cancer (relative to normal bladder samples) using publicly available microarray data-

KDM6A constructs, transfections, RT-PCR, and functional assays

Short hairpin RNA (shRNA) targeting human KDM6A (shKDM6A) and a scrambled shRNA control (shCTL) in nontargeting plasmid plKO.1-puro (Sigma-Aldrich) was used to examine KDM6A in MGHU3 cells. Mammalian expression vectors allowing reexpression of KDM6A (FLAG-KDM6A) or empty control vector (FLAG) were constructed using a modified Gateway Multisite Recombination system (Life Technologies) and transfected into T24T cells. KDM6A depletion and reexpression was validated by quantitative reverse transcriptase-PCR (qRT-PCR). Anchorage-dependent and -independent growth and cell migration was assessed as previously described (14). For in vivo assessments, 5-week-old male NCrnu/nu mice were injected with 2 x 10^6 MGHU3 cells stably expressing shKDM6A or shCTL and were assessed as described in the Supplementary Methods.

Statistical analysis

The Pearson’s correlation coefficient was used to assess pairs of altered genes in 54 U.S. tumors (ARID1A, BAP1, STAG2, KDM6A, and TERT). We used a Fisher exact test or logistic regression for univariate analysis and logistic
regression for multivariate analysis to investigate association of clinical factors with gene mutation status. Nonsynonymous, coding insertions and deletions (indels), UTR, and intronic variants within 10 bp of the intron/exon border were included in the mutation correlation and the clinical association analyses. Statistical analysis was performed using R software (http://cran.r-project.org).

Results
Exome sequencing of 14 bladder tumors from U.S. patients
We subjected 14 primary urothelial bladder tumors to exome sequencing to characterize genomic variants in U.S. patients with bladder cancer (Supplementary Table S1). This yielded an average of >7.6 gigabases per sample and an average mean NGS read depth of 92× (Supplementary Table S2). Grouping NGS-predicted variants by the type of nucleotide change revealed significant enrichment in C>T and G>A changes, similar to previous reports in bladder and other cancers (Fig. 1A, top; refs. 3 and 15). We identified putative somatic variants by removing variants observed at a frequency >1% in the 1000 Genomes Project and variants in known segmental duplications (16). This yielded 9,312 missense, 348 nonsense, 216 SJ, and 653 coding insertion/deletion (indel) candidate variants.

We analyzed matched tumor and normal tissue DNA (Supplementary Table S1) using PCR and Sanger sequencing.

Figure 1. Altered bladder cancer genes. A, 41 bladder cancer genes with somatic nonsynonymous and SJ alterations. Top histogram, NGS predicted variants by nucleotide change for NMI (stage Ta and T1) and MI (stage T2–T4) tumors. Central panel, genes (left) and CRM-DA functions (teal color, see text); asterisk, a proven or likely oncogene; black dot, tumor has ≥1 somatic mutation; histogram, right side, percent of tumors with somatic gene mutation; bold or hatched box, CNV; bottom, tumor IDs (1–14). B, somatic alterations in 5 CRM-DA bladder cancer genes (top) in 40 validation tumors (left side), annotated as in A.
to confirm NGS-predicted variants and identify somatic from germline alterations. Nonsynonymous and SI variants selected for validation were in genes with >1 NGS-predicted alteration, known cancer and related genes, genes with clearly deleterious variants (frameshift and nonsense), and several randomly selected genes. We examined 316 variants and confirmed 228 (72%), 112 were somatic (Supplementary Table S3) and 116 were germline, including 43 novel germline variants (Supplementary Table S4).

Somatic alterations in 4 novel bladder cancer genes

Validated somatic variants were observed in 67 genes, including 10 indels, 22 nonsense, 78 missense, and 2 variants in SIs. Twenty genes were mutated in >1 tumor, including genes known to be mutated in bladder cancer such as FGFR3 (17), TP53 (18), and TSC1 (ref. 19; Fig. 1A, middle; Supplementary Table S3). Eight genes were altered in ≥3 tumors and are referred to as frequently mutated genes (FMG), including previously identified bladder cancer genes, KDM6A and ARIID1A, altered in 4 and 3 tumors, respectively (3). Twenty additional genes with a somatic alteration are also included in Fig. 1A (bottom) based on previous identification as a cancer or cancer-related gene. This selection is further supported by the types of somatic alterations. For example, MLL3 is altered in tumor 6 by a somatic c.C8695T (p.Q2899X) and was altered in 25% of TCGA bladder cancer tumors, and EP300 is altered in tumor 2 by a somatic c.G3052T (p.E1018X) and was altered in 17% of TCGA bladder cancer tumors (20, 21).

To our knowledge, 4 altered genes are novel bladder cancer FMGs when compared with other studies (5): BAP1 located on chromosome (chr) 3p21, chromodomain helicase DNA binding protein 1 (CHD1, chr. 5q21), chromodomain helicase DNA binding protein 1-like (CHD1L, chr. 1q21), and GCN1 general control of amino-acid synthesis 1-like 1 (GCN1L1, chr. 1q24). These genes are of particular interest as they encode proteins that have distinct roles in chromatin remodeling (Fig. 1A, gene names in teal). These FMGs increase the number of bladder cancer genes encoding proteins with CRM-DA functions functions. An average of 4.7 somatic sequence changes to CRM-DA genes occurred in each tumor, range 2–13, indicating a substantial contribution from altered CRM-DA genes to bladder cancer.

Somatic CHD1, CHD1L, and GCN1L1 alterations in bladder cancer

Four alterations in CHD1 were confirmed as somatic (Fig. 1A). Recurrent somatic C>T variants in tumors 3 and 5 altered a highly conserved proline 1684 to serine (p. P1684S) and a somatic C>T in tumor 7 introduced a nonsense codon (p.E272X), truncating most of the 1,710 amino acid (aa) protein (NM_001270). CHD1 encodes a CHD family-related protein that alters chromatin structure and influences transcription via SNF2-related helicase/ATPase and chromatin organization modifier domains (22). Somatic alterations were observed in genes related to CHD1, including a truncation (p.Q728X) of CHD1L and a p.Q1833E in CHD4. Altered expression of CHD1L has been implicated in bladder cancer (23) and germline alterations are associated with congenital anomalies of the kidney and urinary tract (24), suggesting CHD1L is a valid bladder cancer gene. A total of 4 of 14 tumors (29%) showed somatic alteration of CHD genes. CHD2, CHD4, CHD5, and CHD6 were altered in 4%, 3%, 4%, and 7%, respectively, of Chinese patient tumors in a total of 15 of 99 tumors (15%; ref. 5).

Four somatic alterations of GCN1L1 were observed and these may lead to loss of function, as indicated by a single-base deletion in tumor 10 causing a frameshift and truncation after codon 1777 (2671 aa full length, NM_006836; Fig. 1A). Two somatic, synonymous changes were observed previously in 99 Chinese patient tumors (5). The current data indicate, to our knowledge, the first evidence of somatic nonsynonymous GCN1L1 alterations in bladder cancer. GCN1L1 may link AA metabolism to chromatin remodeling and gene expression through roles in regulating GCN2 kinase and as a transcriptional coregulator in mediator complexes (25). Future analyses of CHD1, CHD1L, CHD-related genes, and GCN1L1 in additional bladder cancer tumors are planned to determine accurate somatic mutation frequencies in bladder cancer.

BAP1 and BRCA pathway defects in bladder cancer

Exome sequencing revealed 4 somatic missense variants in BAP1 in 3 tumors (Fig. 1 and Supplementary Table S3). To confirm BAP1 as a bladder cancer gene, PCR and Sanger sequencing was performed on an additional 40 bladder tumors with matched normal tissue from U.S. patients (Supplementary Table S1). Five additional somatic alterations were observed and, in total, BAP1 was altered in 8 of 54 tumors (15%).

The likely functional effects of BAP1 mutations are predominantly loss of function, such as introduction of a stop codon (p.E257X) and 2 somatic changes altering histidine 169 to an arginine (p.H169R) or a glutamine (p.H169Q; Fig. 2A). Histidine 169 has a critical function as the predicted proton donor residue in the ubiquitin hydrolase active site (Uniprot DB). A comparative 3-dimensional (3D) model of BAP1 was generated at ModBase and the structure confirms that p.H169 occupies a critical position directly across from the active site nucleophile, cysteine 91 (Fig. 2B, adapted from ModBase; ref. 11). Both substitutions alter the active site: histidine to an arginine introduces a significantly longer side chain and histidine to a glutamine introduces an uncharged for a positively charged residue. BAP1 was also altered in 2 tumors by recurrent, nonconservative substitutions of a glycine for arginine 718 (p.R718G). Arginine 718 is 1 of 4 charged arginine residues in a highly conserved NLS consisting of AAs 717-722 (Fig. 2C, refs. 11 and 12). Previously published alteration of arginines to alanines in the NLS, including p.R718, completely blocked cytoplasmic to nuclear shuttling of the protein and abolished BAP1 deubiquitination activity. These observations are consistent with deleterious somatic alterations targeting a tumor suppressor in...
bladder cancer, similar to previous observations of BAP1 in other cancers (26).

BAP1 protein binds breast cancer 1 (BRCA1; ref. 27) and loss of function BAP1 mutations may target the BRCA DNA repair pathway, including BRCA1 and breast cancer 2 (BRCA2) (28). This is supported by 16 somatic alterations in BAP1 and 4 BRCA DNA repair pathway genes in 9 of 14 tumors (64%; Fig. 1A and Supplementary Table S3). A tenth tumor was homozygous for a truncating BRCA2 germline allele, totaling a surprising 10 of 14 (71%) bladder cancer tumors with BRCA pathway alterations (Supplementary Table S4). Eight tumors were altered by clearly deleterious mutations, including 4 truncations, and approximately 1 pathway gene per tumor was inactivated (6 tumors). Ataxia telangiectasia mutated (ATM) was altered by 6 somatic sequence changes in 4 tumors, including alteration of a conserved SI (c.5497-1G>C) and a p.Q1636X in tumors 9 and 12, respectively. BRCA1 was altered by somatic missense p.P364A and p.S1286T in 6 tumors and 3, respectively. BRCA2 was altered by a homozygous germline p.K3326X and a somatic p.Q3066X in tumors 7 and 11, respectively. Finally, partner and localizer of BRCA2 (PALB2) was altered by somatic p.S335C and p.Q613X in tumors 6 and 10, respectively. These alterations define a majority of bladder cancer tumors with a common DNA repair deficiency that might be exploited by targeted therapies such as poly (ADP-ribose) polymerase (PARP) inhibitors (28).

BAP1 mutation in kidney tumors is associated with rhabdoid morphology (26) and we examined this phenotype in bladder cancer tumors. Blinded to the BAP1 status, one author (S.M. Lucia) classified a set of 16 H&E-stained tumor sections, 8 with a BAP1 mutation and 8 WT. This revealed papillary features in 6 tumors (Fig. 2D and Supplementary Table S5), of which 5 had a somatic BAP1 alteration. Three tumors with BAP1 mutations did not exhibit papillary features in the tumor section analyzed, although additional tumor sections were not available to examine other regions of these tumors. Our data suggest that papillary features in some bladder tumors are associated with somatic alteration of BAP1.

We compared BAP1 somatic mutations in 54 U.S. patient tumors to 99 Chinese patient tumors (5) and found BAP1 was altered at a significantly greater frequency in the U.S. cohort (15% vs. 1%, P = 0.003). This remained marginally significant after accounting for differences in tumor grade and stage (P = 0.037). To determine if other CRM-DA genes were altered at different frequencies between these cohorts, we sequenced the 3 most frequently mutated CRM-DA bladder cancer genes, ARID1A, KDM6A, and STAG2 (3, 5, 29) in the same set of 40 validation tumors (Fig. 2A and Supplementary Table S1). ARID1A, KDM6A, and STAG2 were altered by somatic sequence changes in 17%, 24%, and 17%, respectively, of 54 U.S. patient tumors (Supplementary Table S3). Cumulatively, the 4 genes were altered in 25 of 54 (46%) of U.S. tumors. Guo and colleagues (5) observed ARID1A, KDM6A, and STAG2 alterations in 15%, 30%, and 11%, respectively, of 99 Chinese patient tumors. The somatic mutation frequencies of ARID1A, KDM6A, and STAG2 were similar and only BAP1 was significantly different. Thus, BAP1 seems to be preferentially altered in U.S. patients with bladder cancer, perhaps because of patient ethnicity, exposure, or lifestyle factors.

Copy number alterations in bladder cancer genes

Exome and Sanger sequencing revealed allelic imbalance at sites of somatic and heterozygous germline sequence variants in tumor versus normal tissue DNA, which could be classified as copy-number variation CNV (Fig. 1, bold boxes). Somatic and heterozygous germline variants with high RSI scores ≥ 0.7 (mutant allele signal/WT + mutant allele signal) are reliable indicators of CNVs in cancer (8, 9). The 41 bladder cancer genes in Fig. 1A were altered by 27 CNVs with an average of 2 per tumor, range 0 to 5. These genes were altered by 100 somatic sequence changes with an average of 7.1/tumor, range 4 to 16. In total, an average of 9.1 somatic alterations/tumor, range 4 to 20, were observed, which were predominantly sequence changes (78%) compared with CNVs (21%). Two or more alterations potentially affecting both alleles of a bladder cancer gene were detected in 30 genes with an average of 2.1 multiply-altered genes/tumor, range 0 to 4. Similar results were observed for 5 genes analyzed in B (Fig. 1B). Somatic alterations (n = 77) were composed of 64 sequence changes (83%) and 13 CNVs (17%), and 17 tumors (43%) displayed at least 1 gene with >1 alteration. These data indicate homozygous loss of function of tumor suppressor genes or homozygous activation of oncogenes affects 2 genes/tumor and indicates potential high priority therapeutic targets.

Rare, deleterious germline alleles in bladder cancer

Exome and targeted sequencing of 54 tumors identified 11 deleterious germline variants resulting in nonsense, frameshift truncation, and SI alterations. Seven alleles
caused frameshift truncations, 3 introduced nonsense codons, and 1 altered a highly conserved 11 nucleotide bold, Supplementary Table S4). Two previously identified cancer genes were altered, BRCA2 (p.K3326X, rs11571833) and RB1 (p.Q846fs, novel). One novel allele in tumor 9 truncated the FGF binding protein 1 (FGFBP1; c.6delG, p.K26fs), potentially indicating a new (likely rare) disease-associated allele in the FGF signaling pathway. Finally, we describe tumors with germline TERT promoter variants, including identical variants confirmed as either germline or somatic in distinct tumors. These data suggest a greater than expected contribution to these supposed sporadic cases of bladder cancer from rare germline variants.

**Somatic and germline alteration of the TERT promoter**

Somatic TERT promoter alterations were recently reported in melanoma, glioma, and in small sample sets from other cancers, including bladder (7, 30, 31). We examined the proximal promoter of TERT in 54 bladder tumors and identified 93 single nucleotide substitutions in 48 of 54 tumors, composed of 29 unique variants (Figs. 1, 3 and Supplementary Table S6). Of these, 42 nucleotide substitutions, consisting of 20 unique variants, were germline in 30 of 54 tumors (56%) and 19 were novel (asterisk; Fig. 3B). The most common variant, c.−245T>C (rs2853669), was observed in 32 of 54 tumors (59%). This variant was confirmed as germline in tumors from 23 patients and as a novel somatic (tumor-specific) change in 9 tumors. Novel variants at 2 other sites (c.−113C>T, and c.−212C>G and c.−212C>T) were also confirmed as both germline and somatic in distinct tumors.

We observed 51 somatic alterations in 37 of 54 tumors (69%) consisting of 11 unique variants, including 4 novel variants, c.−111C>T, −113C>T, −133C>G, and −212C>G (asterisk, Fig. 3B and Supplementary Table S6). Eleven tumors (20%) had >1 somatic alteration and 1 sample (tumor 17) had 4 variants in a cluster, c.−111, −112, −113, and c.−124. Three somatic variants previously reported in other cancers (7, 30, 31) were observed in multiple bladder tumors, c.−124C>T in 27 tumors (50%), c.−245T>C in 9 tumors (17%) tumors, and c.−146C>T in 7 tumors (13%), suggesting these nucleotides are recurrent targets in bladder cancer. Cytosine to thymine alterations comprised 39 of 51 (76%) of the observed somatic TERT promoter alterations.

Similar to a previous report (31), a subset of somatic and germline variants were predicted to create new TFBS (Fig. 3C). Variant c.−113C>T creates a potential c-Rel binding site; variants c.−124C>T, c.−138C>T, and c.−139C>T potential Ets binding sites; variant c.−228A>G, a potential Sp1 binding site; variant c.278G>A, a potential HSF2 binding site, and variant c.−284C>T, a potential Elk-1 binding site. In addition, variants altered sequences known to bind TFs by chromatin immunoprecipitation followed by NGS (ENCODE data tracks in the UCSC Genome Browser; Fig. 3A), including Pol2, TAF1, EGR-1, Myc, Max, Smad3A, and CTCF. Variants c.−110A>T, c.−111C>T, c.−112C>T, c.−113C>T, c.−126C>T, and c.−133C>G altered multiple Sp1 binding sites causing an expected loss of TF binding (32). Finally, 3 promoter variants altered CpG dinucleotides or neighboring residues (±1 bp), including a somatic c.−269G>A, germline c.−306G>A, and germline c.−329C>T. These may affect regulation of promoter methylation.

**Association of mutations with patient and tumor variables**

Next we examined 5 genes for which we had somatic mutation status in 54 tumors, ARID1A, BAP1, KDM6A, STAG2, and TERT, for correlations in somatic mutations and for associations between mutation status and clinical factors. We found only BAP1 and KDM6A mutations were correlated (P = 0.017; Supplementary Table S7). Somatic alteration of the TERT promoter occurred in tumors both with and without mutations in each of the other genes. These data indicate likely independent contributions of the mutations in these 5 genes to the tumor cell phenotype. No significant associations between the mutation status of the 5 genes and clinical factors were observed (not shown).

**Network analysis reveals the importance of KDM6A in bladder cancer**

To better understand the potential interactions between altered bladder cancer genes, we performed network analysis on genes with confirmed somatic mutations in this study and a recent study of 99 Chinese bladder tumor exomes (5). This identified several robust interacting networks and biologic functional groups (Supplementary Table S8). The highest scoring network (P < 10−46) was composed of 34 mutated genes known to bind Ubiquitin C (UBC; Supplementary Fig. S1). The second highest scoring network (P < 10−46) contained proteins encoded by the third through the fifth most mutated genes in the Chinese cohort (ARID1A, CREBBP, and EP300; ref. 5; Supplementary Fig. S2). Genes encoding enzymes associated with the deubiquitinylation pathway, including BAP1, which is mutated in this study (BAP1, DUB, USP21, USP26, USP31, USP34, USP36, and USP48), and DNA methylation (ARID4B, CHD4, CHD3, MTA, and SIN3A), were well represented in this network. The third highest scoring network (P < 10−42) contained TP53 along with 4 genes involved in DNA methylation (DNMT1, DNMT3A, MLL3, and MLL5) and multiple genes associated with chromosomal structural elements and posttranslational modifiers of histones H3 and H4 (Fig. 4A).

KDM6A is frequently mutated in both United States and Chinese tumors and alterations are significantly correlated with somatic BAP1 alterations. KDM6A has limited protein–protein interaction data, however, it could be included in the third highest scoring network because this network is the only 1 of the 3 that contains proteins known to interact with KDM6A. Inclusion of KDM6A in a network different from BAP1 makes sense because mutations in these genes are correlated and therefore they likely contribute different
selective advantages to bladder cancer cells. We also included the RB1 tumor suppressor in this network, based on previously described relationships with TP53 and KDM6A (33). We found that KDM6A interacts with several other proteins encoded by genes mutated in Chinese tumors. KDM6A directly binds to MLL3, CSPG4, and SMARCA4 (BRG1; refs. 34 and 35) and regulates RB1 expression (33). MLL3, RB1, and SMARCA4 are known tumor suppressors.

Figure 3. TERT promoter variants in bladder cancer. A, The proximal TERT locus in the UCSC Genome Browser is shown with HG19 genomic coordinates (Hg19), TERT isoforms (TERT transcripts), TFBS from ENCODE ChIP-seq, the amplicon location (Amplicon BLAT), and a CpG-rich region (CpG island). B, The TERT promoter amplicon (5’, top left, to 3’, bottom right) is shown with germline variants (above) and somatic variants (below) the genomic reference sequence (solid line). Variant annotation is based on the TERT coding strand. Frequencies of variants (n = 54, in parentheses), novel variants (asterisk), and variants in new TFBS (underlined, see details in Fig. 3C) are indicated. C, TFBS in the TERT promoter (bold horizontal line, center) are shown relative to the transcription start site (TSS) and the protein-coding start codon (ATG): , Sp1; , E-box; and , Ets TFBS. New TFBS created by novel (single underline) and previously identified variants (double underline) are shown in the enlarged regions (boxed).
whereas CSPG4 is thought to be oncogenic (35, 39). Using 3 publicly available microarray datasets (Supplementary Table S9), we identified genes that were consistently up- or downregulated in tumors in at least 2 datasets (FDR < 5%) and highlighted them on the mutation network (Fig. 4A). In addition to being a known tumor suppressor interacting with KDM6A, SMARCA4 expression was upregulated in tumors in all 3 cohorts. KDM6A indirectly interacts with several proteins consistently deregulated in bladder cancer, such as histone H3, which is required for EGF induced transformation (40). Network analysis revealed that KDM6A interacts with the products of genes that harbor bladder cancer driver mutations and with genes consistently differentially expressed in bladder cancer, suggesting its central importance as a driver of bladder cancer. We also used Cytoscape (www.cytoscape.org) to plot these genes in 3D space using the comutational rate and the frequency of mutation as parameters in our model. This puts KDM6A as the most central and well-connected gene in this mutational network (Fig. 4B).

**Functional relevance of KDM6A in bladder cancer**

KDM6A is frequently mutated in bladder cancer and is important in signaling networks as we show above. Therefore, we developed a gene mutation signature for KDM6A (13), where a high KDM6A signature score corresponded to a likely somatic mutation. We examined 3 patient cohorts (Supplementary Table S9) and found higher scores corresponded to NMII bladder cancer stratified patients with a more favorable prognosis. KDM6A signature scores are higher in bladder tumor samples compared with normal urothelial cells (Fig. 5), indicating the signature likely detected altered KDM6A-related pathways in bladder cancer. Although these studies suggest that KDM6A is a driver of bladder cancer and has prognostic value, the direct functional role of KDM6A in bladder cancer has not yet been experimentally established.

Hence we determined the role of KDM6A in shaping the cancer cell phenotype using 2 well-established human bladder cancer models, T24T (14) and MGHU3 (41). These tumor-derived cell lines were selected based on KDM6A mutation status. MGHU3 was WT, and T24T had a homozygous G>T nonsense mutation introducing a premature stop codon (p.E895X, NM_021140, 1401 aa full length). The stop codon truncated 506 C-terminus AAs (36% of the full length protein) including the catalytic jumonji C domain (see Materials and Methods). shRNA and cDNA constructs were used to deplete and overexpress WT KDM6A in MGHU3 and T24T, respectively. The effect of these treatments on KDM6A expression was verified by qPCR (Fig. 6A). KDM6A depletion in MGHU3 enhanced anchorage independent growth ($P = 0.04$) and cell migration...
GCN1L1 (2%) Alteration of endometrial (4%), stomach (2%), and lung cancers matin remodeling and/or regulation. Overexpression of CHD1L in bladder cancer because of their influence on chromatin structure and transcription (22). Overexpression of CHD1L in MGHU13 led to significantly enhanced tumor growth (P = 0.002), confirming the functional relevance of intact KDM6A as a tumor growth inhibitor (Fig. 6D). The deleterious mutations observed in tumors in this and other studies (3, 5) and the functional data presented here indicate KDM6A is a frequently mutated gene encoding a tumor suppressor in bladder cancer.

Discussion

This study contributes to the catalog of genes altered in human bladder cancer by evaluating 54 tumors from U.S. patients. We identified 41 genes altered by somatic variants that may be relevant to disease, including 4 genes, CHD1, CHD1L, GCN1L1, and BAP1, not previously reported as mutated in bladder cancer. Interestingly, all 4 impact chromatin remodeling and/or regulation.

CHD1-family gene alterations may play an important role in bladder cancer because of their influence on chromatin structure and transcription (22). Overexpression of CHD1L has been implicated in bladder cancer (23) and germline alterations are associated with congenital anomalies of the kidney and urinary tract (24), indicating a likely role in bladder development. CHD1 somatic alterations have been reported in prostate (42) and other cancers (21, 43) and a review of the COSMIC DB shows CHD1L is altered in endometrial (4%), stomach (2%), and lung cancers (2%). Alteration of GCN1L1 may target its role in AA metabolism and excretion (44). The COSMIC DB shows GCN1L1 is mutated in 4% to 7% of endometrial, cervical, urinary tract (bladder), colon, lung, and stomach cancers. We plan to examine additional bladder cancer tumors to confirm accurate mutation frequencies for CHD1, CHD1L, and GCN1L1.

BAP1 acts to remodel chromatin via an ubiquitin hydrolase catalytic function that removes ubiquitin from histone H2A. Somatic and germline BAP1 alterations have been observed in melanoma, mesothelioma, and kidney cancers (45–47). Somatic alterations of BAP1 define a subtype of clear cell kidney cancer characterized by papillary features (26). Interestingly, review of bladder tumor samples revealed that papillary features were more common in bladder tumors with mutant BAP1, indicating similar phenotypic characteristics between BAP1-mutant bladder and kidney tumors. BAP1 mutations are correlated with KDM6A alterations, suggesting these alterations may provide complementary advantages to tumor cells, similar to co-occurrence of PBRM1 and SETD2 mutations in renal cell carcinoma (48). Interestingly, BAP1 mutations were complementary to alterations in genes encoding proteins of the BRCA DNA repair pathway (ATM, BRCA1, BRCA2, and PALB2). Components of this pathway are altered in Fanconi anemia, an inherited, autosomal recessive disease characterized by leukemia and an increased susceptibility to multiple types of cancer. The pathway regulates the cellular responses to DNA damage and, via ATM, associated cell-cycle checkpoints. To our knowledge, frequent alteration of the BRCA pathway in bladder cancer has not been previously reported. Mutations in BRCA pathway genes may indicate tumors that are deficient in DNA repair and therefore vulnerable to DNA lesions created by chemotherapeutic drugs (28), especially if combined with PARP (49) inhibitors.

A high frequency of somatic TERT promoter alterations was recently reported in several cancers (7, 30, 31). Although we found germline and somatic alterations in bladder cancer that had not been previously identified, somatic TERT promoter alterations were not associated with alterations in other bladder cancer genes or with tumor stage or grade. The variants observed may affect the TERT promoter in several ways. TERT expression is known to be regulated by methylation and hydroxymethylation of promoter CpGs (50, 51). Several variants altered CpGs or neighboring nucleotides, suggesting potential disruption of epigenetic regulation of TERT. In addition, ENCODE data show germline and somatic variants altered DNA sequences known to bind TFs. TF binding may also be altered by variants predicted to create new TF binding sites,

(P < 0.001) but not monolayer growth (Fig. 6B–D). In T24T cells, KDM6A overexpression diminished anchorage independent growth (P = 0.02) and cell migration (P = 0.055) but not monolayer growth (Fig. 6B–D). In vivo, depletion of KDM6A in MGHU13 led to significantly enhanced tumor growth (P = 0.002), confirming the functional relevance of intact KDM6A as a tumor growth inhibitor (Fig. 6D). The deleterious mutations observed in tumors in this and other studies (3, 5) and the functional data presented here indicate KDM6A is a frequently mutated gene encoding a tumor suppressor in bladder cancer.
such as c. –113C>T, which creates a consensus sequence for c-Rel. Hence, germline and somatic variants may work in concert to alter TERT gene expression.

It is not clear why certain cancer genes vary in mutation frequency across patient cohorts with the same disease. When compared with bladder tumors from Chinese patients, BAP1 is mutated at a higher frequency in tumors from U.S. patients, although the mutation frequencies of KDM6A, ARID1A, and STAG2 were similar between these cohorts. Thus, BAP1 and perhaps BRCA pathway genes may represent genes targeted for alteration because of specific ethnic, lifestyle, or geographic differences. Given its frequent and similar mutation frequency in both patient cohorts, we sought to determine the functional and prognostic role of KDM6A in human bladder cancer. 

In vitro and in vivo experiments examining KDM6A depletion and overexpression in bladder tumor cells support a role for KDM6A as a suppressor of tumor growth and cell migration.

Taken together, our work provides further insights on the genomic landscape of bladder cancer while providing clues to possible prognostic (KDM6A status signature score) and companion biomarkers (BAP1 and BRCA pathway gene mutations), suggesting novel therapeutic strategies (PARP inhibitors combined with DNA damaging agents) that may be effective against bladder cancer.

Figure 6. KDM6A loss drives the bladder cancer phenotype. KDM6A WT MGHU3 cells were treated with shRNA targeting KDM6A (shKDM6A) or a scrambled shRNA (shCTL). These were compared with KDM6A-mutant T24T cells transiently reexpressing FLAG-tagged KDM6A (FLAG-KDM6A) or empty FLAG vector (FLAG). A, relative KDM6A mRNA expression by qPCR. B, anchorage-independent growth assay (n = 6 wells/line). C, transwell cell migration (n = 4 wells/line). D, monolayer growth of cells (left) assessed by CYquant fluorescence assay (n = 4 wells/line) and subcutaneous tumor growth (right, n = 20 mice/line). Results are shown as the mean ± SEM. P values were calculated using a Student’s t test on the final day of the assay. Assay details are described or referenced in Materials and Methods.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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