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Original Article

CDKN1A/p21^{WAF1}, RB1, ARID1A, FLG, and HRNR mutation patterns provide insights into urinary tract environmental exposure carcinogenesis and potential treatment strategies

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Abstract: Bladder carcinoma has a 6% 5-year survival-rate for metastatic disease, with poorly understood links between genetic and environmental drivers of disease development, progression, and treatment response. Rhode Island has among the highest annual age-adjusted incidence rate of bladder cancer at 26.0/100,000, compared to 20.0 in the US, with a paucity of known driver genes for targeted therapies or predictive biomarkers. Bladder carcinomas have the highest frequency of alterations in CDKN1A/p21^{WAF1} (10%) across all cancer types analyzed in The Cancer Genome Atlas (TCGA) PanCancer Atlas Studies, displaying a predominance of truncating mutations (86%). We found that lung carcinomas lack CDKN1A truncating mutations, despite the shared role of tobacco as a risk factor for bladder cancer. Bladder carcinomas also have the highest frequency of RB1 alterations in TCGA (25%). We find that chromophobe renal cell carcinomas with CDKN1A and RB1 mutations are 100% truncating. Analysis of 1,868 bladder tumors demonstrated that truncating CDKN1A mutations co-occur with truncating RB1 mutations, suggesting an environmental exposure signature. Moreover, we found that HRNR and FLG mutations are enriched in tumors with CDKN1A alteration, suggesting potential novel roles in promoting bladder tumorigenesis. Association of HRNR with AKT activation offers possible therapeutic avenues, and FLG may provide insight into carcinogen exposure within the bladder. We suggest that because APOBEC mutations largely shape the bladder cancer mutational landscape, these events likely contribute to dysfunctional DNA repair genes, leading to frameshifts and the predominance of truncations in CDKN1A, RB1, ARID1A, or other drivers. We propose that patients with co-occurrence of CDKN1A and RB1 truncations may display enhanced responsiveness to targeted therapies combining cisplatin with ATR, ATM, CHK1, and CHK2 inhibitors, expanding therapeutic options for patients in need of improved precision treatments.

Keywords: Bladder cancer, chromophobe renal cell carcinoma, environmental carcinogenesis, cisplatin, checkpoint kinases, truncating mutations

Introduction

In the United States in 2021, there will be an estimated 83,730 new cases of urinary bladder carcinoma and 17,200 deaths [1]. A strong male prevalence is observed, with almost 75% of all cases occurring in men [2], and tumors most commonly arise in the seventh decade of life [3]. This disease can present as non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC), or as a metastatic form, and each has different molecular drivers. Through whole-transcriptome mRNA profiling, bladder cancer was revealed to have one of the highest mutation rates of any cancer sequenced to date, following only lung cancer and melanoma [4].

Next-generation sequencing technologies have helped to elucidate the genomic complexity of

bladder carcinomas. Overall, somatic gene alterations are most common in pathways related to p53, the cell cycle, and RAS-PI3K, in addition to epigenetic modifications [5]. Many tumors display missense or truncating mutations in TP53, driving loss-of-function. Additionally, homologous deletions and truncations are common in cell cycle genes, resulting in the inactivation of genes such as CDKN2A, CDKN2B, RB1, and CDKN1A. Gain-of-function mutations are predominantly seen in FGFR3, PIK3CA, ERBB2, and ERBB3 [6], promoting tumorigenesis. Moreover, bladder cancer exhibits significant epigenetic dysregulation at the level of DNA methylation [7]. DNA hypermethylation is linked to the silencing of a number of tumor suppressor genes, including TP53, RB1, CDKN2A, and CDH1, and is associated with more aggressive disease [8]. Bladder cancer also has a higher mutational load than most cancers in chromatin remodeling genes, such as inactivating mutations in ARID1A, a SWI/SNF chromatin remodeling subunit, and the histone demethylase KDM6A [5]. This suggests that loss of epigenetic regulation may also help promote bladder tumorigenesis.

Furthermore, next-generation sequencing has helped to identify specific molecular subgroups. NMIBC shows a predominance of deletions in CDKN2A, and mutations in FGFR3, PI3K, and TERT have been identified as early drivers of malignancy [5]. Among all cancer types, MIBC has the highest enrichment of APOBEC-specific mutations, with most APOBEC-specific mutations found in the gene promoter of TERT [9]. Tumors with APOBEC enrichment, termed APOBEC-high, are more likely to have mutations in DNA damage response genes (TP53, ATR, BRCA2) and chromatin regulatory genes (ARID1A, MLL, MLL3) [10]. By contrast, APOBEC-low tumors are more likely to have mutations in FGR3 and KRAS. Yet, despite continuing efforts to identify genetic drivers of disease, precision therapies for bladder cancer remain scarce.

Mainstay treatments for bladder cancer currently depend on whether tumors present with muscle invasiveness. NMIBC is treated with endoscopic resection and adjuvant immunotherapy with Bacillus Calmette-Guerin (BCG), but patients who fail to respond to BCG subsequently have limited therapeutic options [11]. MIBC, in contrast, is treated with more aggressive therapies, including radical cystectomy, a cisplatin-based combination neoadjuvant chemotherapy regimen, specifically cisplatin-gemcitabine [12], and radiation. Nevertheless, the benefits of chemotherapies are limited to a subset of patients, and the inability to predict responsiveness remains a major challenge.

Our previous work has demonstrated that sensitivity to cisplatin-based chemotherapies is induced by inactivation of CDKN1A, the gene encoding the cyclin dependent kinase inhibitor $p21^{WAF1}$ [13]. Cisplatin induces DNA adducts, which halts cell proliferation and activates the DNA damage response [14]. Cells deficient in p21 are less able to repair cisplatin-induced DNA adducts, resulting in a greater extent of DNA damage after p21 loss. Loss of p21 also prevents CDK activation, driving progression through the cell cycle without efficient repair of DNA damage. This results in procession down an apoptotic pathway and helps to explain sensitization to cisplatin [13]. Therefore, mutation in CDKN1A has the potential to serve as a candidate biomarker to predict chemotherapy responsiveness. Moreover, CDKN1A has been implicated as a prognostic marker in bladder cancer, as lower p21 expression has been associated with advanced pathologic stage, tumor grade, and lower overall survival [15]. Further characterization of additional genes dysregulated in concordance with CDKN1A is needed to better elucidate the mechanisms driving disease and to enhance options for precision therapies.

In addition to knowledge of particular genes involved in tumorigenesis, it has been demonstrated that exposure to a number of environmental agents and chemicals are closely associated with an increased risk of developing bladder cancer. The most notable risk factor is occupational exposure to aromatic amines, including 2-naphtylamine, 4-aminobiphenyl, and benzidine, and 4,4'-methylenebis (2-chloroaniline); these chemicals are found in the products of chemical, dye, and rubber industries, as well as in fungicides, plastics, metals, and motor vehicle exhaust [16]. Moreover, cigarette smoking is a known primary risk factor for bladder cancer, resulting in a threefold higher risk of developing disease in smokers [17]. Carcinogenesis induced by smoking is attributed to the presence of chemicals in tobacco smoke, particularly 2-naphtylamine and 4-aminobiphenyl. There is also strong evidence that

links the development of bladder cancer with exposure to arsenic in drinking water. Despite understanding that these environmental carcinogens contribute to tumorigenesis, further work is needed to elucidate the particular genes affected by these chemicals as well as the mechanisms that drive transformation.

Current efforts focus on further defining the mutational landscape of bladder tumors to enhance molecular characterization as well as to identify actionable subgroups. Importantly, predictors of chemosensitivity are needed to avoid preventable toxicity and the delay of life-saving radical cystectomies in patients who will prove to be resistant [18]. Evaluation of DNA and RNA from patients' urine has recently been approved as a diagnostic marker [19], and new panels include the measurement of gene expression levels, sequence variations, histone modifications, and DNA methylation. Such advancements are continuing to drive the development of precision therapies.

Because further work is needed to identify concordant biomarkers driving bladder cancer, we sought to analyze genes dysregulated alongside CDKN1A. Aside from identifying the prevalence of truncating mutations in genes such as CDKN1A, RB1, and ARID1A, we also propose novel genes that may contribute to tumorigenesis in bladder carcinomas, HRNR and FLG, which are enriched for alterations in tumors that also harbor CDKN1A mutations. Moreover, we discovered that a similar predominance of truncations exists in chromophobe renal cell carcinomas, suggesting that DNA damaging agents may also be a therapeutic option for patients with this disease. By further elucidating co-occurring vulnerabilities in bladder tumors with CDKN1A mutations, we propose a number of novel avenues to explore the efficacy of potential targeted therapies in combination with standard-of-care cisplatin to help improve patient outcomes in qualifying molecular subgroups.

Methods

TCGA PanCancer Atlas Studies analyzed on cBioPortal included the following: Adrenocortical Carcinoma, Cholangiocarcinoma, Bladder Urothelial Carcinoma, Colorectal Adenocarcinoma, Breast Invasive Carcinoma, Brain Lower Grade Glioma, Glioblastoma Multiforme, Cervical Squamous Cell Carcinoma, Esophageal Adenocarcinoma, Stomach Adenocarcinoma, Uveal Melanoma, Head and Neck Squamous Cell Carcinoma, Kidney Renal Clear Cell Carcinoma, Kidney Chromophobe, Kidney Renal Papillary Cell Carcinoma, Liver Hepatocellular Carcinoma, Lung Adenocarcinoma, Lung Squamous Cell Carcinoma, Diffuse Large B-Cell Lymphoma, Acute Myeloid Leukemia, Ovarian Serous Cystadenocarcinoma, Pancreatic Adenocarcinoma, Mesothelioma, Prostate Adenocarcinoma, Skin Cutaneous Melanoma, Pheochromocytoma and Paraganglioma, Sarcoma, Testicular Germ Cell Tumors, Thymoma, Thyroid Carcinoma, Uterine Corpus Endometrial Carcinoma, Uterine Carcinosarcoma.

Studies analyzed on cBioPortal included the following; bladder urothelial carcinomas: Bladder Cancer (MSK/TCGA, 2020; https://www.cbioportal.org/study/summary?id=blca_msk_tcga_2020), Bladder Cancer [20], Bladder Cancer [21], Bladder Cancer [22], Bladder Cancer [8], Bladder Urothelial Carcinoma [23], Bladder Urothelial Carcinoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/), Non-muscle Invasive Bladder Cancer [24], Urothelial Carcinoma [25]; upper tract urothelial carcinomas: Upper Tract Urothelial Cancer [26], Upper Tract Urothelial Carcinoma [27], Upper Tract Urothelial Carcinoma [28], Upper Tract Urothelial Carcinoma [29], Upper Tract Urothelial Carcinoma PDX [29]; Skin Cutaneous Melanoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/), Skin Cutaneous Melanoma [30]; kidney chromophobe: Kidney Chromophobe (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/); ovarian serous cystadenocarcinoma: Ovarian Serous Cystadenocarcinoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/); lung adenocarcinoma: Lung Adenocarcinoma [31], Lung Adenocarcinoma [32], Lung Adenocarcinoma [33], Lung Adenocarcinoma [34], Lung Adenocarcinoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/), Lung Adenocarcinoma [35], Non-Small Cell Cancer [36]; lung squamous cell carcinoma: Lung Squamous Cell Carcinoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/); sarcoma: Sarcoma [37], Sarcoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/); uterine carcinosarcoma: Uterine Carcinosarcoma [38], Uterine Carcinosarcoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.

org/); uterine corpus endometrial carcinoma: Uterine Corpus Endometrial Carcinoma (TCGA, Firehose Legacy; https://gdac.broadinstitute.org/).

Results

Bladder urothelial carcinomas display a high frequency of CDKN1A ($p21^{WAF1}$) truncating mutations

Because early work suggested that mutations in CDKN1A in cancer are rare, we asked how the alteration frequency of CDKN1A in bladder urothelial carcinomas compared to a number of other cancer types. We examined the TCGA PanCancer Atlas Studies, and found that 10.46% of all bladder urothelial carcinomas screened had a CDKN1A alteration (**Figure 1A**), the highest among all cancer types included. The most common event among these alterations was mutations. While smoking is a known risk factor for the development of bladder carcinoma, only 1.44% of lung squamous cell carcinomas and 1.06% of lung adenocarcinomas displayed CDKN1A alterations, suggesting disparate driver mechanisms in bladder and lung carcinomas despite exposure to a common carcinogen.

We next sought to investigate the CDKN1A mutational landscape to better characterize particular mutations. We found that 85.54% of all bladder carcinoma CDKN1A mutations were truncating (**Figure 1B**), with seven being the largest number of mutations at a single location and representing an amino acid change of R84Vfs*40/3Pfs*3/3Lfs*6/3Lfs*61/4Gfs*2 (**Figure 1C**). Among the bladder carcinoma studies included in cBioPortal, the highest CDKN1A alteration frequency was 23.53% (Supplementary Figure 1A). A similar mutational pattern was also depicted in upper tract urothelial carcinomas, as 78.95% of CDKN1A mutations were truncating. We also analyzed the frequencies of particular mutations of the other cancer types most strongly enriched for CDKN1A alterations in TCGA, and, interestingly, found that 100% of mutations in kidney chromophobes were truncating. However, there was no enrichment of truncations in skin cutaneous melanoma or ovarian serous cystadenocarcinoma. Moreover, the mutational pattern seen in bladder carcinoma did not extend to that of lung cancers, despite the tobacco carcinogenesis common to both, as both lung adenocarcinomas and lung squamous cell carcinomas displayed a predominance of CDKN1A missense mutations and lacked truncating mutations entirely.

Among tumors with a CDKN1A deletion, we next asked whether there was a predominance of homozygous or heterozygous allelic loss to better understand the potential mechanisms driving sensitivity to DNA damaging agents like cisplatin. Through analysis of copy-number data for CDKN1A, we found that 0.2% of deletions were deep, representing homozygous loss, and 16% were shallow, representing heterozygous loss (**Figure 1D**). This suggests that loss of a single CDKN1A allele may be sufficient to drive the subsequent phenotype of cisplatin sensitivity, with additional mechanisms in effect dependent upon mutations in other genes.

In order to better understand whether the presence of a CDKN1A mutation could serve as a biomarker for clinical prognosis, we compared the difference in overall survival for tumors with and without a CDKN1A alteration. Those that harbored a CDKN1A mutation displayed a trend toward a worse outcome with 17.97 median months of overall survival in comparison to 32.00 for those lacking a mutation (Supplementary Figure 1B), though the difference was not statistically significant. Together, these results indicate that the presence of CDKN1A inactivating truncating mutations in bladder carcinomas is likely an aberrant driver event in tumorigenesis and can also serve as a predictive biomarker for poorer clinical outcomes.

Given the significant enrichment of truncating mutations in CDKN1A, we wondered whether TP53 would also show a unique enrichment of truncations in bladder carcinomas compared to other cancer types. We found that, unlike CDKN1A, only 25.03% of TP53 mutations in bladder urothelial carcinomas were truncating, and that TP53 truncating mutations were similarly present across a number of cancer types (Supplementary Figure 2A). Moreover, in contrast to CDKN1A, the most common mutation in TP53 was missense, with 48 being the largest number of mutations at a single location and representing an amino acid change of R248Q/W/P/G (Supplementary Figure 2B).

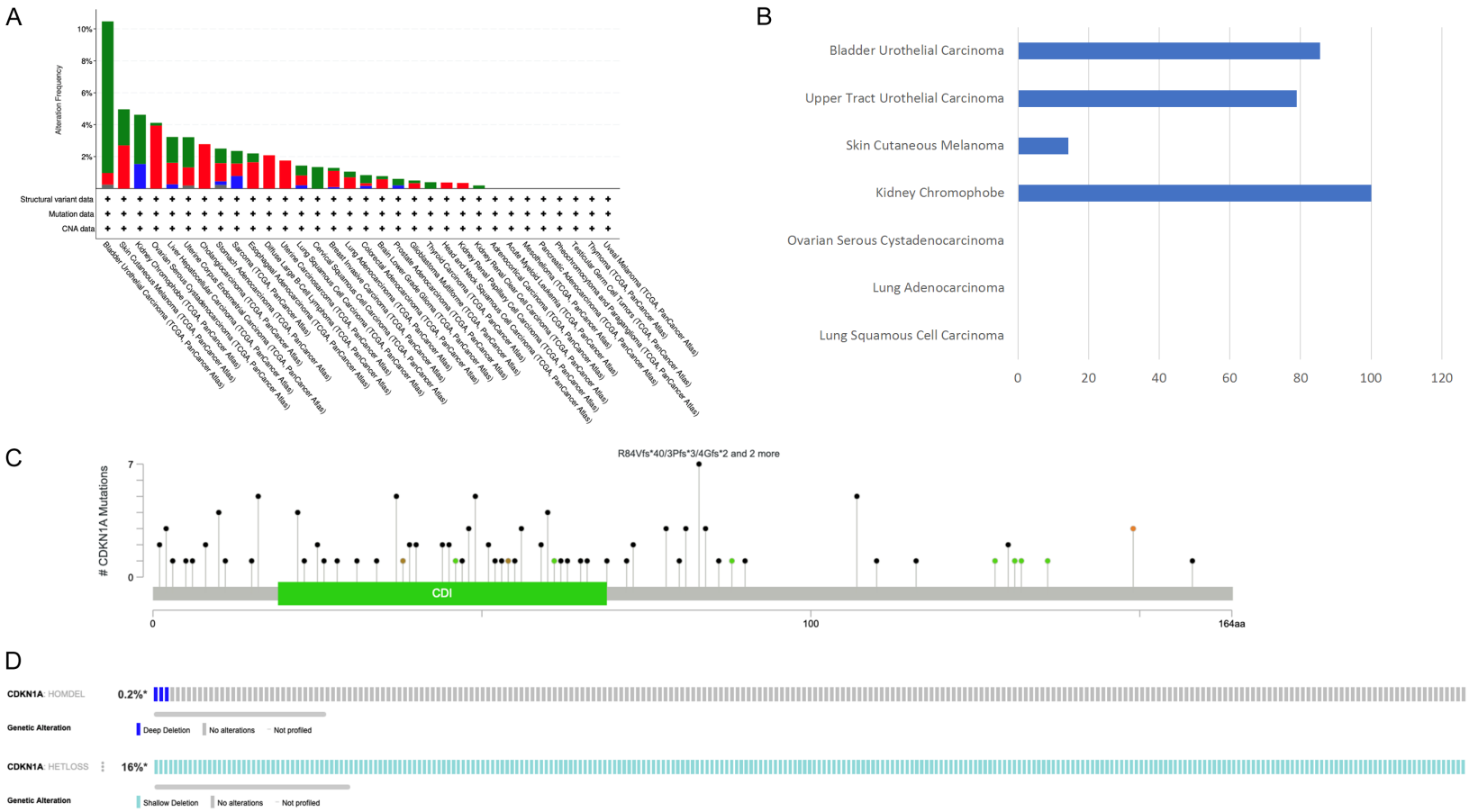

Figure 1. Bladder urothelial carcinomas display a high frequency of CDKN1A truncating mutations. A. CDKN1A alteration frequencies in TCGA PanCancer Atlas Studies. Alterations include mutations (green), amplifications (red), deep deletions (blue), and multiple alterations (gray). B. Percentages of CDKN1A truncating mutations across the cancer types most strongly enriched for CDKN1A alterations in TCGA, in addition to lung cancers included in TCGA. C. Schematic of CDKN1A mutations. Depicted are driver truncating mutations (black, 142 total), VUS missense mutations (green, 21 total), VUS in-frame mutations (brown, 2 total), and driver splice mutations (orange, 1 total). D. Percentages of CDKN1A deletions that are homozygous (deep deletions, dark blue, top) or heterozygous (shallow deletions, light blue, bottom).

These findings suggest that there are particular genes subject to truncating mutations in bladder carcinomas, rather than a generalized pattern seen among all members of the p53 pathway. For example, other TP53 target genes, such as BBC3 and TP53I3, did not show an enrichment of truncations. When analyzing the TP53 status of tumors with a CDKN1A alteration, we found that 60.39% were wildtype for TP53 while 39.61% had a TP53 alteration (Supplementary Figure 2C). We propose that patients with the combination of a CDKN1A alteration and wildtype TP53 are the molecular subgroup likely to have enhanced responsiveness to cisplatin.

APOBEC, mismatch excision repair, and homologous recombination gene mutations are enriched in tumors that also harbor a CDKN1A mutation

Because the APOBEC mutational signature is the predominant pattern in muscle-invasive bladder cancer, we asked whether tumors with CDKN1A alterations were enriched for any mutations in genes that are a part of this signature. We found enrichment for alteration events in the APOBEC genes PIK3CA, BRCA2, KMT2C, and ARID1A in tumors that also harbored a CDKN1A alteration (**Figure 2A**), though none of these enrichments were statistically significant. Moreover, we found statistically significant enrichments in alterations in MSH6 and PMS1, genes involved in nucleotide excision repair, in tumors that also had a CDKN1A mutation (**Figure 2B**). Finally, there were statistically significant enrichments in alterations in BRCA1 and PALB2, and non-statistically significant enrichments in alterations in ATM, CDK12, FANCC, and RAD51C, genes involved in homologous recombination, in tumors that also had a CDKN1A mutation (**Figure 2C**). These findings suggest that aberrant activity in a number of genes involved in DNA repair pathways may drive frameshifts that, in turn, result in downstream truncating mutations in genes like CDKN1A.

Because the APOBEC mutational signature displays a high proportion of C>T and C>G mutations, we sought to determine whether we saw a similar enrichment of this pattern in tumors with CDKN1A truncating mutations. Among bladder tumors included in cBioPortal with nonsense mutations, 54.55% harbored a C>T or C>G mutation. Moreover, among tumors with frameshift mutations, 20% of deletions were C nucleotides, and 57.89% of insertions were T or G nucleotides. These findings offer further evidence to support that CDKN1A truncations likely occur downstream of alterations in APOBEC genes.

We also aimed to further investigate the alteration events of genes included in the APOBEC mutational signature across the multiple bladder carcinoma studies in cBioPortal. Consistent with previous findings, we found that the most frequent alterations in TP53, PIK3CA, ATR, BRCA2, KMT2A, KMT2C, and ARID1A were mutations (Supplementary Figure 3A). The strongest predominance of these mutations was in muscle-invasive carcinomas, with the exception of PIK3CA and ARID1A. Interestingly, ARID1A displayed a mutational pattern similar to CDKN1A, as 66.52% of all mutations were truncating. This suggests a potential role of dysregulation of chromatin regulatory genes downstream of the APOBEC mutational landscape.

Because APOBEC genes are known to play roles in DNA repair and chromatin regulation, we also asked whether bladder carcinomas had enrichments in mutations for genes involved in mismatch excision repair or homologous recombination. We found that the most prevalent alterations in MSH2 and MSH6 were mutations. In contrast, the most predominant alterations in MLH1 and PMS2 were amplifications (Supplementary Figure 3B). Among homologous repair genes, BRCA1, PALB2, ATM, CDK12, and FANCC all showed a predominance of mutations, while RAD51C displayed a prevalence of amplifications (Supplementary Figure 3C). Together, these results suggest that inefficiency in DNA repair in bladder carcinomas is driven by compounded mutations in APOBEC, mismatch repair, and homologous recombination genes, likely driving frameshift events downstream.

RB1, TERT, MUC16, and HRNR are the genes with the highest overall alteration frequencies and are enriched in tumors that also harbor a CDKN1A alteration

In order to nominate potential candidates for combination therapies and better understand

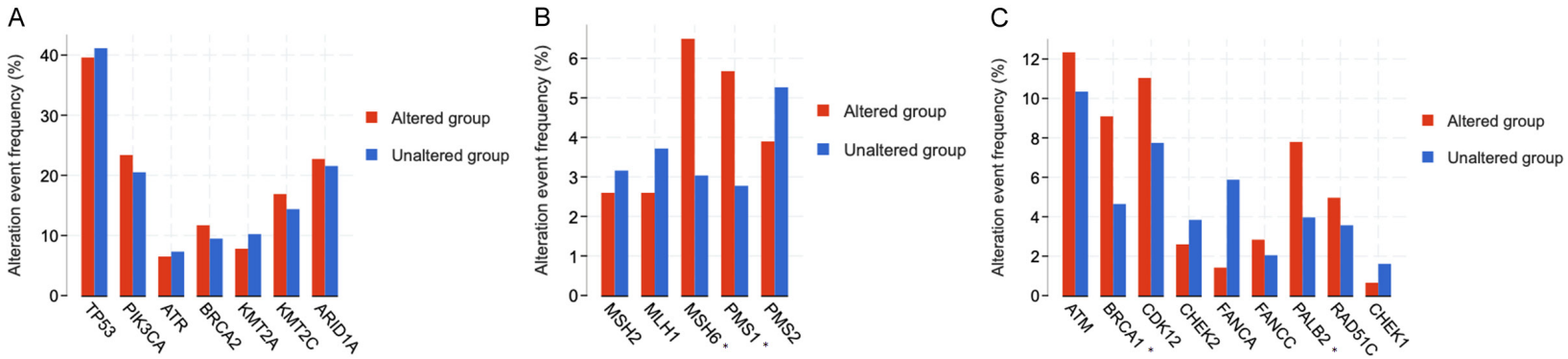

Figure 2. Mutations in APOBEC, mismatch repair, and homologous recombination genes are enriched in tumors that also harbor a CDKN1A alteration. A. Frequencies of alteration events in genes included in the APOBEC mutational landscape in tumors that harbor a CDKN1A alteration (red) versus those that do not (blue). B. Frequencies of alteration events in mismatch repair genes in tumors that harbor a CDKN1A alteration (red) versus those that do not (blue). C. Frequencies of alteration events in homologous recombination genes in tumors that harbor a CDKN1A alteration (red) versus those that do not (blue). A star next to the gene name indicates that the gene in statistically significantly enriched in the altered group.

the mechanism of action of bladder carcinogens, we first analyzed genes with the highest overall frequency of alterations that were also enriched in tumors with CDKN1A mutations. Interestingly, one such gene was RB1 (**Figure 3A**), which also had the highest frequency of alterations in bladder carcinomas, 16.3%, in comparison to all other cancer types included in TCGA (**Figure 4A**). Upon further analyzing RB1 alteration events, we found a predominance of mutations, with 26.47% as the highest mutation rate among all studies included in cBioPortal (Supplementary Figure 4A). Strikingly, 79.07% of these mutations were truncating (**Figure 4B**), with eight being the largest number of mutations at a single location and representing an amino acid change of X405_splice (**Figure 4C**). The predominance of truncating mutations in RB1 recapitulates the pattern seen in both CDKN1A and ARID1A. Among the cancer types in TCGA with the highest frequency of RB1 alterations, there was a broad enrichment of truncating mutations in RB1. RB1 truncations were particularly enriched in sarcomas and, unlike what was seen in CDKN1A, RB1 truncations were present in both lung adenocarcinomas and lung squamous cell carcinomas, suggesting a potential link between tobacco smoke and bladder tumorigenesis through dysregulation of RB1.

Similar to the analysis performed to further investigate CDKN1A deletions, we asked whether there was an enrichment of homozygous or heterozygous allelic loss among tumors with an RB1 deletion. Copy-number data for RB1 revealed that 6% of deletions were deep, indicating homozygous loss, and 19% were shallow, indicating heterozygous loss (**Figure 4D**). Importantly, the predominance of heterozygous deletions in both CDKN1A and RB1 points to a potential mechanism of haploinsufficiency. Among tumors harboring a CDKN1A alteration, 30.56% also had an alteration in RB1. Most importantly from a therapeutic standpoint, among the tumors with alterations in both CDKN1A and RB1, 40.31% also had TP53 wild-type status (Supplementary Figure 4C); we propose that patients with this specific molecular profile are most likely to respond to cisplatin. Of the bladder urothelial carcinoma samples included in cBioPortal, 103 had a CDKN1A alteration, 268 had an RB1 alteration, and 44 had both CDKN1A and RB1 co-occurring alterations (**Figure 4E**). Given the statistically significant tendency for CDKN1A and RB1 alterations to co-occur, this offers a promising avenue for novel targeted therapies in combination with cisplatin.

In addition to RB1, TERT, MUC16, and HRNR were among the genes with the highest overall alteration frequencies that were statistically significantly enriched in tumors also harboring a CDKN1A alteration. TERT promoter mutations and MUC16 alterations have both previously been shown to contribute to bladder tumorigenesis. Interestingly, TERT is mutated in a striking 72.38% of non-muscle invasive bladder cancers (Supplementary Figure 5A), suggesting that its role in promoting tumorigenesis may be unique to this molecular subtype. Moreover, MUC16 is mutated in as high as 38% of bladder urothelial carcinomas. On the contrary, HRNR is yet to be implicated in bladder tumorigenesis, but bladder urothelial carcinomas have the fourth highest rate of HRNR alterations across all cancer types screened in TCGA (Supplementary Figure 5C). The most frequent alterations were an equal split between mutations and amplifications (Supplementary Figure 5B). Interestingly, these findings may highlight a novel role of HRNR in driving bladder urothelial carcinomas.

RAB44 is among the genes with the most significant P-values enriched in tumors that also have a CDKN1A alteration

We next sought to analyze the set of genes with the most significant *P*-values for enrichment in alterations in tumors that also have a CDKN1A mutation. One such gene among this set was RAB44 (**Figure 3B**), which has previously been demonstrated to play a role in promoting bladder tumorigenesis. The predominance of alteration events in RAB44 in bladder carcinomas are amplifications (Supplementary Figure 5E), suggesting that this gene may be a viable therapeutic target.

Bladder tumors with a CDKN1A alteration are enriched for mutations in FLG

Because of the large number of environmental carcinogens that have been implicated in bladder tumorigenesis, we asked whether any genes that function in maintaining the epidermal barrier showed an enrichment of muta-

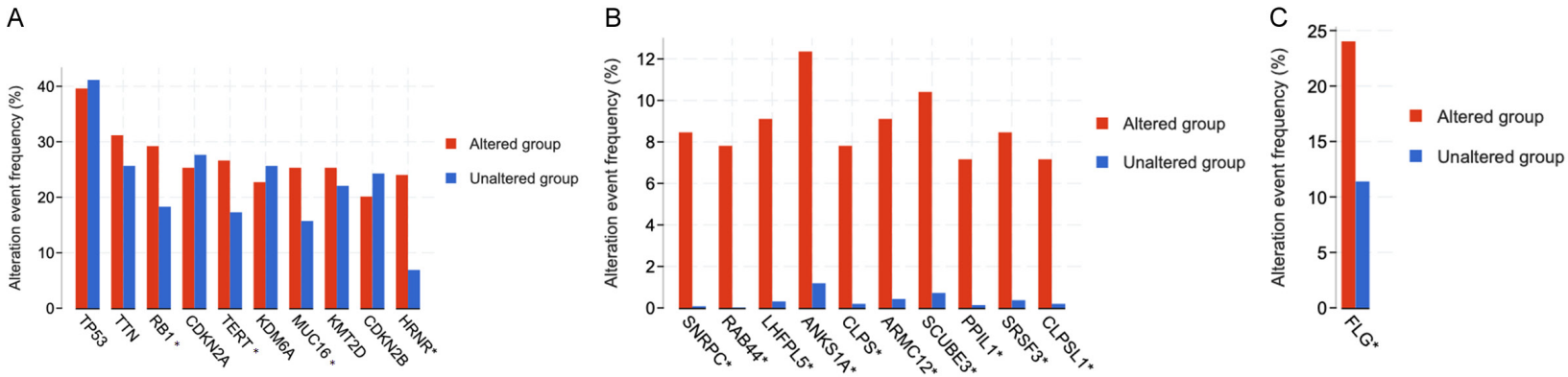

Figure 3. RB1, TERT, MUC16, RAB44, HRNR, and FLG are enriched for alterations in tumors that also harbor a CDKN1A alteration. A. Frequencies of alteration events in genes with the highest frequency of alterations of any group in tumors that harbor a CDKN1A alteration (red) versus those that do not (blue). B. Frequencies of alteration events in genes with the most statistically significant p vales in tumors that harbor a CDKN1A alteration (red) versus those that do not (blue). C. Frequency of alteration events in FLG in tumors that harbor a CDKN1A alteration (red) versus those that do not (blue). A star next to the gene name indicates that the gene in statistically significantly enriched in the altered group.

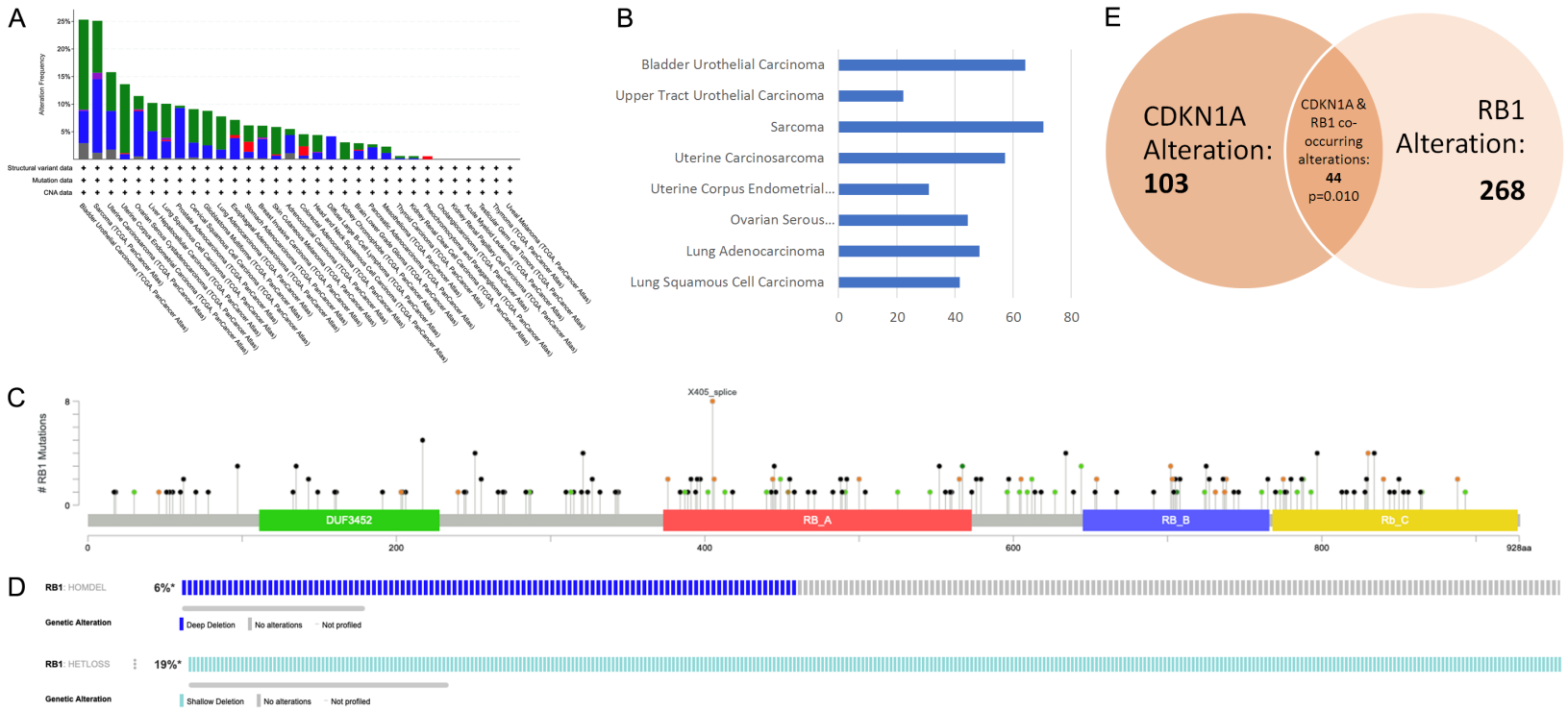

Figure 4. Bladder urothelial carcinomas display a high frequency of RB1 truncating mutations. A. RB1 alteration frequencies in TCGA PanCancer Atlas Studies. Alterations include mutations (green), amplifications (red), deep deletions (blue), and multiple alterations (gray). B. Percentages of RB1 truncating mutations across the cancer types most strongly enriched for RB1 alterations in TCGA, in addition to lung cancers included in TCGA. C. Venn diagram depicting tumor samples that harbored a CDKN1A alteration only (103/1425, left), an RB1 alteration only (268/1425, right), or co-occurring alterations (44/1425, center). Log2 odds ratio 0.687, *P*-value = 0.010, tendency for co-occurrence. D. Schematic of RB1 mutations. Depicted are driver truncating mutations (black, 201 total), VUS missense mutations (light green, 46 total), driver missense mutations (dark green, 5 total), driver splice mutations (orange, 58 total), VUS in-frame mutations (brown, 2 total), and driver SV/fusion mutations (purple, 1 total). E. Percentages of RB1 deletions that are homozygous (deep deletions, dark blue, top) or heterozygous (shallow deletions, light blue, bottom).

tions in tumors that also harbor a CDKN1A mutation. One such gene, FLG, had a statistically significant enrichment for alterations in tumors with a CDKN1A mutation (**Figure 3C**), and mutations were the most common event (Supplementary Figure 5F). FLG is also yet to be implicated in bladder carcinogenesis, but bladder urothelial carcinomas have the sixth highest rate of FLG alterations across all cancer types screened in TCGA (Supplementary Figure 5G). In addition to a potential novel role of FLG in promoting bladder cancer, mutations in FLG may also offer insight into a possible mechanism behind environmental exposure to carcinogens.

Discussion

We report a novel genomic signature defined by the prevalence of truncating mutations in both CDKN1A and RB1 in bladder carcinomas, with a statistically significant tendency of these alterations to co-occur. These unusual gene mutation signatures likely reflect unique pathways of carcinogen exposure through the environment with accumulation of carcinogens or their metabolites in the bladder. It has previously been demonstrated that CDKN1A mutations render cells unable to halt the cell cycle and efficiently repair DNA damage, leading to apoptosis. These CDKN1A truncating mutations therefore not only drive sensitivity to cisplatin [13], but also offer the possibility for combination therapies that additionally target RB1. RB1 knockout has been shown to enhance bladder tumorigenesis both in vitro and in vivo [39], and it has been demonstrated that RB-deficient tumor cells have a greater dependence on CHK1 [40], a key regulator of the DNA damage response (DDR) which enables DNA repair and allows for cell cycle progression. Bladder tumors with deficiency of the tumor suppressor RB1 have defects in the G1 checkpoint, driving genomic instability.

We propose that tumors with co-occurring CDKN1A and RB1 loss-of-function truncations may show enhanced sensitivity to a spectrum of precision therapies with ATR, ATM, CHK1, and CHK2 inhibitors. Preclinical studies have demonstrated that CHK1 inhibitors in combination with cisplatin [41] or gemcitabine [42] potentiate the anticancer activity of these chemotherapeutic drugs. Inhibition of the DDR drives checkpoint abrogation, inhibition of DNA repair, and induction of cell death. Additional work remains to be done in order to determine how these combination therapies enhance the efficacy of cisplatin in patients with CDKN1A alterations, RB1 alterations, or co-occurring alterations, and whether these treatments could be viable therapeutic options in the clinic for patients with qualifying genomic alterations.

Moreover, investigation is needed to unravel the molecular pathways by which environmental carcinogens cause bladder cancer at high rates, such as in Rhode Island and other New England States, as well as to establish strategies for prevention. Our findings warrant further experimentation to determine whether the combination of checkpoint kinase inhibitors with cisplatin will offer more efficacious personalized therapeutics for patients with tumors that harbor cell cycle checkpoint defects.

Relationships between APOBEC and truncating mutational patterns in CDKN1A and RB1 in bladder carcinomas

Of the bladder tumors in TCGA, 80% display the APOBEC mutational signature [10]. The enrichment for mutations in genes that are part of the APOBEC mutational profile in tumors that also harbor CDKN1A mutations further compounds inefficiency in DNA repair, and, importantly, offers a number of targets for precision therapies.

Mutations in the APOBEC gene BRCA2 have been correlated with heritable risks for urothelial carcinomas, as it has been demonstrated that there are significantly higher rates of germline pathogenic variants in BRCA2 compared to cancer-free controls [43]. Interestingly, a rare variant in BRCA2 has been associated with an increased risk of developing both urinary tract and lung cancers [44]. Here, we show that tumors with CDKN1A mutations are enriched for mutations in BRCA1 and BRCA2, both of which contribute to DNA repair and transcriptional regulation in response to DNA damage [45]. This suggests that combination therapy with PARP inhibitors, which preferentially kill BRCA-mutated cancer cells [46], may benefit a subgroup of patients with this particular mutational landscape.

APOBEC activity has also been identified as a key driver of PIK3CA mutagenesis, a gene which we demonstrate to be preferentially

enriched for alterations in tumors with CDKN1A mutations. Activating mutations in PIK3CA, which encodes the catalytic subunit of phosphatidylinositol 3-kinase involved in the PI3K/AKT signaling pathway, are common oncogenic drivers of bladder carcinogenesis. This suggests that patients with PIK3CA mutations may benefit from PI3K-targeted therapies, including PI3K, mTOR, and AKT inhibitors [47], in combination with cisplatin-based therapy.

ARID1A is another gene that is part of the APOBEC mutational landscape, which we show to also be preferentially enriched for mutations in tumors that harbor CDKN1A alterations. ARID1A is a subunit of the SWI/SNF complex, which plays a role in ATP-dependent chromatin remodeling, thereby influencing transcriptional accessibility and modulating DNA repair [48]. In tumors with ARID1A mutations, EZH2 inhibition is synthetic lethal, suppressing cell growth and promoting apoptosis [49]. Bladder tumors with ARID1A deficiencies have previously been shown to be sensitive to the small molecule EZH2 inhibitor GSK-126. Here, we demonstrate that ARID1A mutations in bladder carcinomas are predominantly inactivating truncating mutations, helping to explain sensitivity to EZH2 inhibitors and offering support for the combination of cisplatin and EZH2 inhibitors. Moreover, because mutations in ARID1A have been shown to confer sensitivity to pan-HDAC inhibitors [50], our findings offer additional evidence for the repurposing of pan-HDAC inhibitors for patients whose mutational profiles fall within this subgroup.

RAB44, TERT, MUC16, HRNR, and FLG mutations are enriched in bladder cancers with CDKN1A alterations

The enrichment of mutations in a number of genes, including those that we propose to have novel roles in promoting bladder cancer, in tumors that also harbor a CDKN1A mutation offers new options for therapeutic intervention. First, the Ras oncogene related protein RAB44, a Rab GTPase, has previously been shown to form an oncogenic fusion protein with CDKN1A [51]. Because RAB44 is not expressed in all normal tissue types and the fusion protein has a relatively high prevalence in bladder cancer, this specificity suggests that therapies targeting RAB44 may be a clinical option for patients with these fusion events.

Additionally, TERT, an important element of telomerase expression, was highly enriched in tumors with a CDKN1A alteration. TERT promoter mutations are the most common somatic lesion in bladder cancer and have been demonstrated to be a predictor of both poor survival and disease recurrence [52]. The resulting increased expression of telomerase downstream of TERT promoter mutations offers an attractive target for therapeutic intervention. Therefore, tumors that harbor a CDKN1A alteration may be particularly sensitive to combination therapies with cisplatin and small molecule inhibitors targeting telomere- and telomerase-associated proteins.

Moreover, MUC16, a type of Type 1 transmembrane mucin, was enriched in tumors that also have a CDKN1A alteration. MUC16 has been shown to play a role in angiogenesis as well as mediating metastasis in advanced bladder cancer [53]. As a result, MUC16 alteration in the presence of CDKN1A alteration may serve as a predictive biomarker for clinical prognosis. Excitingly, MUC16 mutation has been associated with an enhanced response to immune checkpoint inhibitors in patients with solid tumors [54], suggesting that immunotherapies may be a viable therapeutic option for patients with co-occurring CDKN1A and MUC16 mutations.

Another gene that we discovered to be strongly enriched in bladder tumors with CDKN1A mutations was hornerin (HRNR). Hornerin is a member of the S100 calcium-binding protein family, which is involved in the regulation of transcription factors, cell proliferation, differentiation, and death [55]. Though HRNR is yet to be implicated in bladder carcinogenesis, its overexpression has been demonstrated in hepatocellular carcinoma tumor progression and is correlated with poor prognosis in HCC [56]. HRNR is necessary to promote AKT phosphorylation, which is required for its activation, and is essential for metastatic pathways. Therefore, we propose that AKT inhibitors may be a potential therapeutic option in combination with cisplatin for patients that harbor HRNR mutations.

From a mechanistic perspective, we found that tumors with CDKN1A mutations are also strongly enriched for mutations in the filaggrin gene (FLG), which encodes a protein product

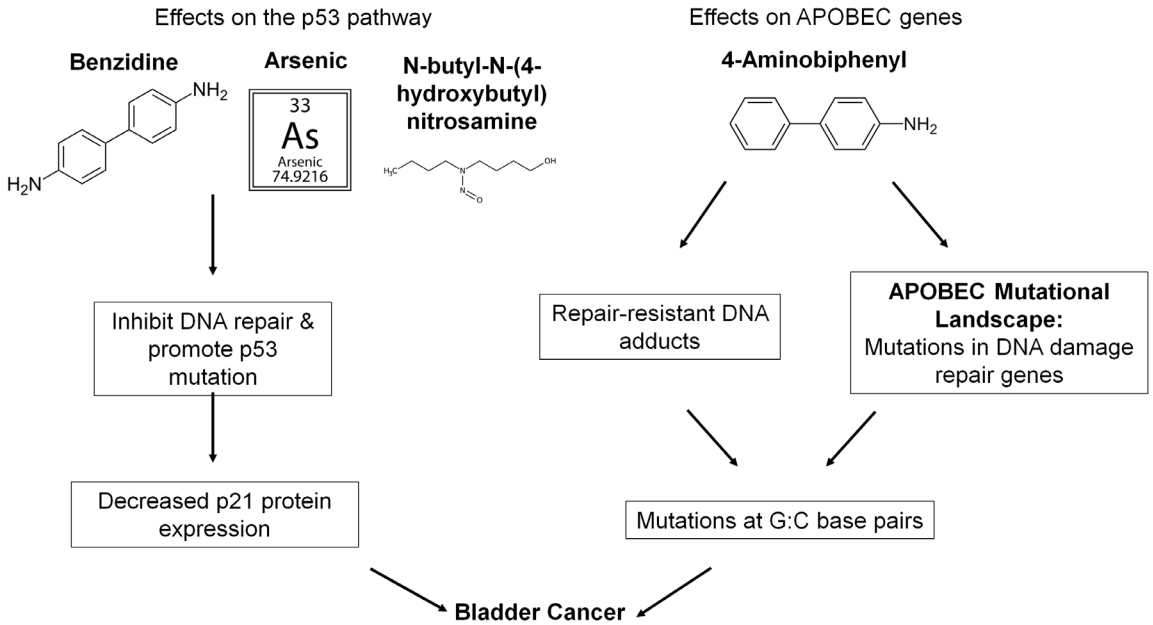

Figure 5. Genomic and environmental carcinogenic mechanisms converge to promote bladder tumorigenesis. Schematic depicting two potential parallel processes that likely act in concordance to promote tumor progression in bladder urothelial carcinomas.

that plays a role in both structural and physiological functions in the skin [57]. Importantly, FLG plays a role in protecting the skin against the uptake of chemicals upon dermal exposure. It is likely that patients that harbor a mutation in these tumors also have a total loss-of-function mutation in the FLG gene. Importantly, dermal exposure to aromatic amines and polycyclic aromatic hydrocarbons is a known risk factor for bladder cancer [58]. An enrichment of FLG mutations in tumors with CDKN1A mutations suggests that these patients may also have an impaired skin barrier function. Resultantly, there is likely a subsequent enhanced absorption of chemicals [59], leading to more profound impacts on DNA and driving carcinogenesis. Activation of the aryl hydrocarbon receptor has been shown to restore FLG expression in atopic dermatitis [60]; further experimentation is needed to determine whether activation of this receptor can also restore FLG expression in bladder carcinomas.

Emerging insights into bladder cancer environmental carcinogenesis from CDKN1A mutational patterns

Rhode Island has for years had the greatest incidence of bladder cancer in the United States among both men and women, a statistic driven both by cigarette smoking and occupational exposures [61]. In the 1950s, Rhode Island exceeded the national average in cigarette smoking, and given the long latency period of bladder cancer development, this historical tobacco use may play a role in recent elevated rates. Moreover, the long history of New England in the textile industry helps to explain the occupation-related exposure to carcinogens, which also has a latency period of 20 years or more. Together, the delayed effects of these historical exposures continue to be seen currently, and Rhode Island is in particularly desperate need of a better understanding of the mechanisms by which carcinogens promote bladder malignancies.

Multiple environmental carcinogens are known to drive bladder tumorigenesis, and a number of these have direct effects on TP53, CDKN1A, and their signaling pathway (**Figure 5**). A thiolated arsenic metabolite, dimethylmonothioarsinic acid, has been shown to cause a decrease in both p21 and p53 protein expression, accompanied by an increase in DNA damage and intracellular hydroxyl radicals [62]. Beyond interfering with the DNA damage response, arsenic has also been shown to result in a decrease in RB1 phosphorylation [63], thereby also disrupting cell cycle regulation. Moreover, N-butyl-N-(4-hydroxybutyl)nitrosamine, an N-nitrosamine, is a compound that has been

identified as a carcinogen specific to bladder cancer in animal studies. Upon treatment with this N-nitrosamine, there has been a demonstrated decrease in p21 protein expression [64], suggesting that lower p21 expression is a potential biomarker for tumorigenesis.

Additionally, 4-Aminobiphenyl (4-ABP) is an aromatic amine generated predominantly from cigarette smoking, and its metabolites have been shown to form repair-resistant DNA adducts. It has previously been demonstrated that 82.9% of mutations induced by 4-ABP occurred at G:C base pairs [54]; as previously stated, a majority of APOBEC mutations are C>G, suggesting that 4-ABP may play a role in the mutagenesis of APOBEC genes. Moreover, a dose-dependent response has been demonstrated between 4-ABP and impaired DNA repair capacity [65]. 4-ABP preferentially forms adducts at two specific codons within the TP53 gene [66]; interestingly, mutations at these codons rarely occur in lung cancer. This specificity of 4-ABP for unique TP53 codons can help explain the TP53 mutational spectrum seen in bladder cancer, and points to potential downstream dysregulation of CDKN1A.

The mechanism of action of benzidine, a known bladder carcinogen, may offer another route for a targeted therapy. Benzidine's structure as an aromatic amine allows it to act as an intercalating agent, likely leading to downstream frameshifts and thereby promoting carcinogenesis. Benzidine has been shown to interact with DNA through both minor groove binding and partial intercalation [67]. Moreover, benzidine has been found to downregulate p21 mRNA levels as well as decrease p21 protein levels [68], provoking the transition of cells from G1 to S and G2. Upon treatment with a MAPK inhibitor, the effects of benzidine on p21 were suppressed. Interestingly, exposure of normal urothelial cells to smoke, a known bladder carcinogen, has been shown to drive MAPK activation [69]. Together, these findings offer support for a combination therapy of cisplatin and MAPK inhibitors, which have previously been shown to induce apoptosis in bladder cancer cell lines [70].

High frequency of CDKN1A and RB1 truncating mutations in chromophobe renal cell carcinomas

We demonstrate a novel mutational profile in kidney chromophobes. In addition to bladder and upper tract urothelial carcinomas, we found that kidney chromophobes are also strongly enriched for CDKN1A and RB1 truncations. Further investigation is needed to determine whether other cancer types, in addition to these, display a similar mutational profile. Given the proximity of the kidneys to the bladder anatomically, this indicates a potential preference of organs involved in urine processing for the enrichment of CDKN1A and RB1 truncations. Importantly, our findings suggest that patients with kidney chromophobes, particularly those with metastatic disease who currently have limited treatment options [71], may benefit from cisplatin-based therapies or other DNA damaging agents. Additional investigation is needed to determine whether patients with kidney chromophobes display enhanced sensitivity to cisplatin.

Moreover, patients with kidney chromophobes with a CDKN1A or an RB1 alteration display a trend toward poorer survival, suggesting that dysregulation of these genes may also serve as prognostic biomarkers for this cancer type. If other cancers beyond bladder, upper tract urothelial carcinomas, and kidney chromophobe tumors do indeed display enrichments of these truncations, this could expand options for precision therapies, as these tumors are likely to also be sensitive to DNA damaging agents and, potentially, the combination therapies described above.

Additionally, it has been shown that 18% of TCGA samples have a strong APOBEC mutation signature [72]. Therefore, further analysis is needed to determine whether other cancer types with predominance of this mutational landscape, such as breast, cervical, and head and neck [4], are also marked by the prevalence of downstream truncating mutations in CDKN1A and RB1. This would provide further insight into the potential mechanisms driving these truncations and, in turn, may help predict sensitivity to cisplatin-based therapies and other DNA-damaging agents.

Implications of truncating mutations in CDKN1A and RB1 for therapeutics of bladder cancer and renal cell chromophobe carcinomas

The findings reported here identify a novel mutational profile in bladder carcinomas. We

reveal a high prevalence of inactivating truncating mutations in CDKN1A, RB1, and ARID1A, likely acting downstream of the APOBEC mutational landscape. We propose a mechanism whereby upstream mutations in APOBEC genes and other genes involved in DNA repair processes drive frameshifts and, in turn, downstream truncating mutations arise. Interestingly, 4-ABP, and potentially other environmental carcinogens, may play a role in driving mutations in APOBEC genes. In addition, a number of environmental carcinogens act on the p53 signaling pathway, resulting in decreased levels of p21 mRNA levels and protein expression downstream. The two parallel processes of dysregulation at the level of the p53 pathway and alterations among APOBEC genes likely converge to promote bladder tumorigenesis.

Due to increases in therapeutic resistance, the classification of patients into distinct molecular subgroups is needed in order to enhance responsiveness to treatment. Previous work has demonstrated that patients who exhibited a better response to neoadjuvant chemotherapy had alterations in one or more of the three DNA repair genes ATM, RB1, and FANCC [73]. Here, we propose a novel genomic signature that may predict chemotherapy sensitivity, as we suggest that patients with co-occurring truncating mutations in CDKN1A and RB1 who also retain wildtype TP53 status are likely to respond most favorably to cisplatin-based therapies and other DNA-damaging agents. Additionally, a prior study suggested that p53 status, as measured by mRNA expression, is a predictor of de novo and induced chemoresistance [74]. Under normal circumstances, p53 will activate the cell cycle checkpoint, increasing CDKN1A expression and, in turn, this promotes DNA damage-induced apoptosis. However, in the presence of CDKN1A alterations, p53's ability to trigger the checkpoint is ineffective, driving sensitivity to cisplatin. Further work is needed to determine whether patients with both CDKN1A and RB1 alterations coupled with wildtype TP53 status display improved clinical benefit.

Additional investigation is required to determine whether homozygous deletions in CDKN1A and RB1 render sensitivity to cisplatin to the same extent as heterozygous deletions. Moreover, further studies are needed to determine whether there is a co-occurrence of CDKN1A mutation and heterozygous CDKN1A allelic deletion in individual tumor samples, as this would result in biallelic loss. Because we found a predominance of heterozygous allelic loss in both CDKN1A and RB1, we propose a potential mechanism of haploinsufficiency, whereby loss of both alleles of CDKN1A is not necessary for the resultant phenotype of sensitivity to cisplatin if co-occurrence of heterozygous loss of both CKND1A and RB1 is present. Our findings offer support for the molecular testing of patients prior to receiving chemotherapy to select for those most likely to respond to treatments and to therefore increase the likelihood of survival.

Most importantly, these findings offer insights into pathways of bladder cancer carcinogenesis through unique truncating mutational signatures, and the potential for a wide range of novel innovative clinical therapies by targeting a number of actionable genes most frequently mutated in tumors that also harbor CDKN1A alterations. Future studies will further explore the effects of these genetic alterations on sensitivity to cisplatin and the proposed combination therapies in pre-clinical systems. Because treatment options are limited for patients with bladder carcinomas, these findings offer support to investigate the potential of checkpoint kinase inhibitors in combination with cisplatin-based therapies both in vitro and in vivo, with hope of future translation into effective personalized clinical therapeutic options.

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Disclosure of conflict of interest

None.

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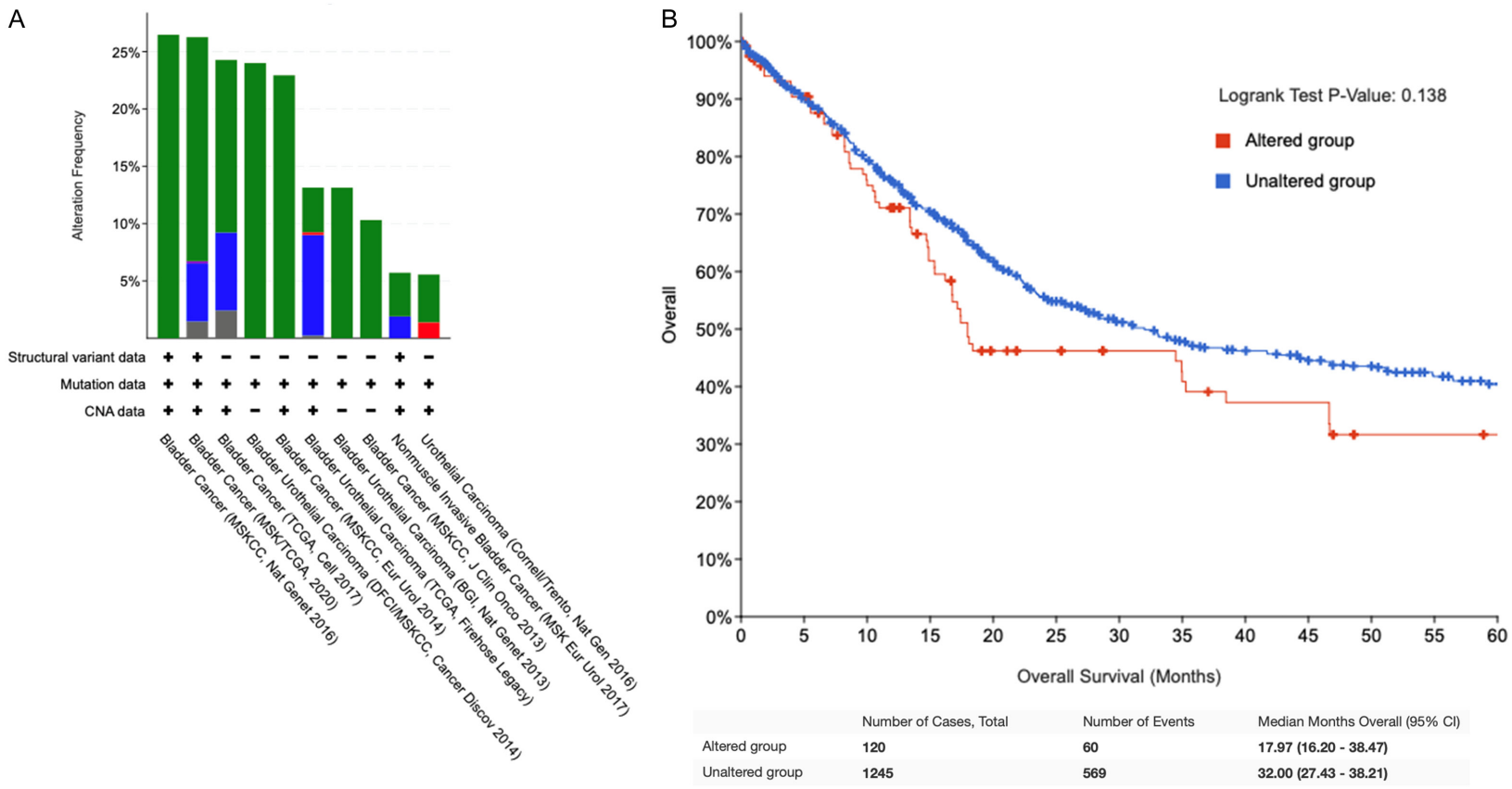
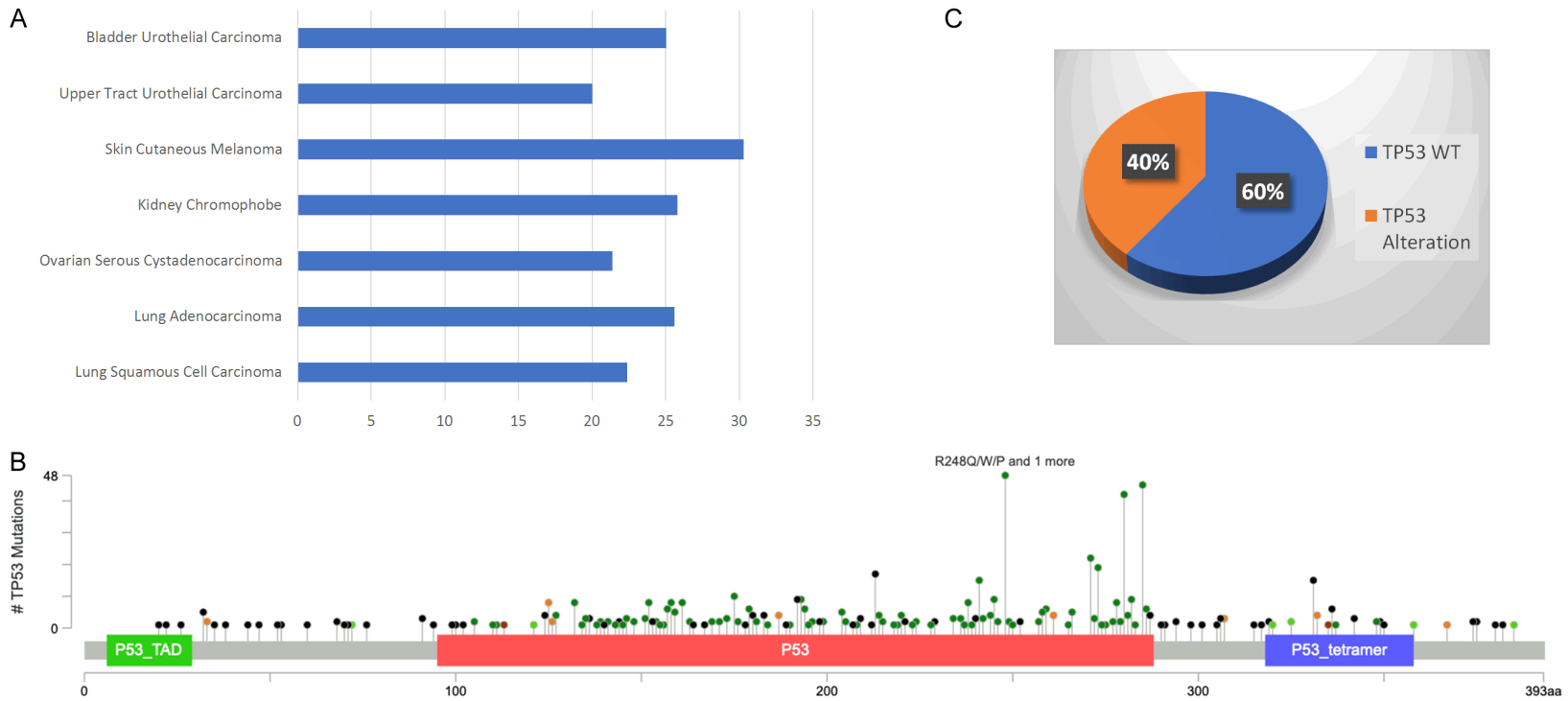
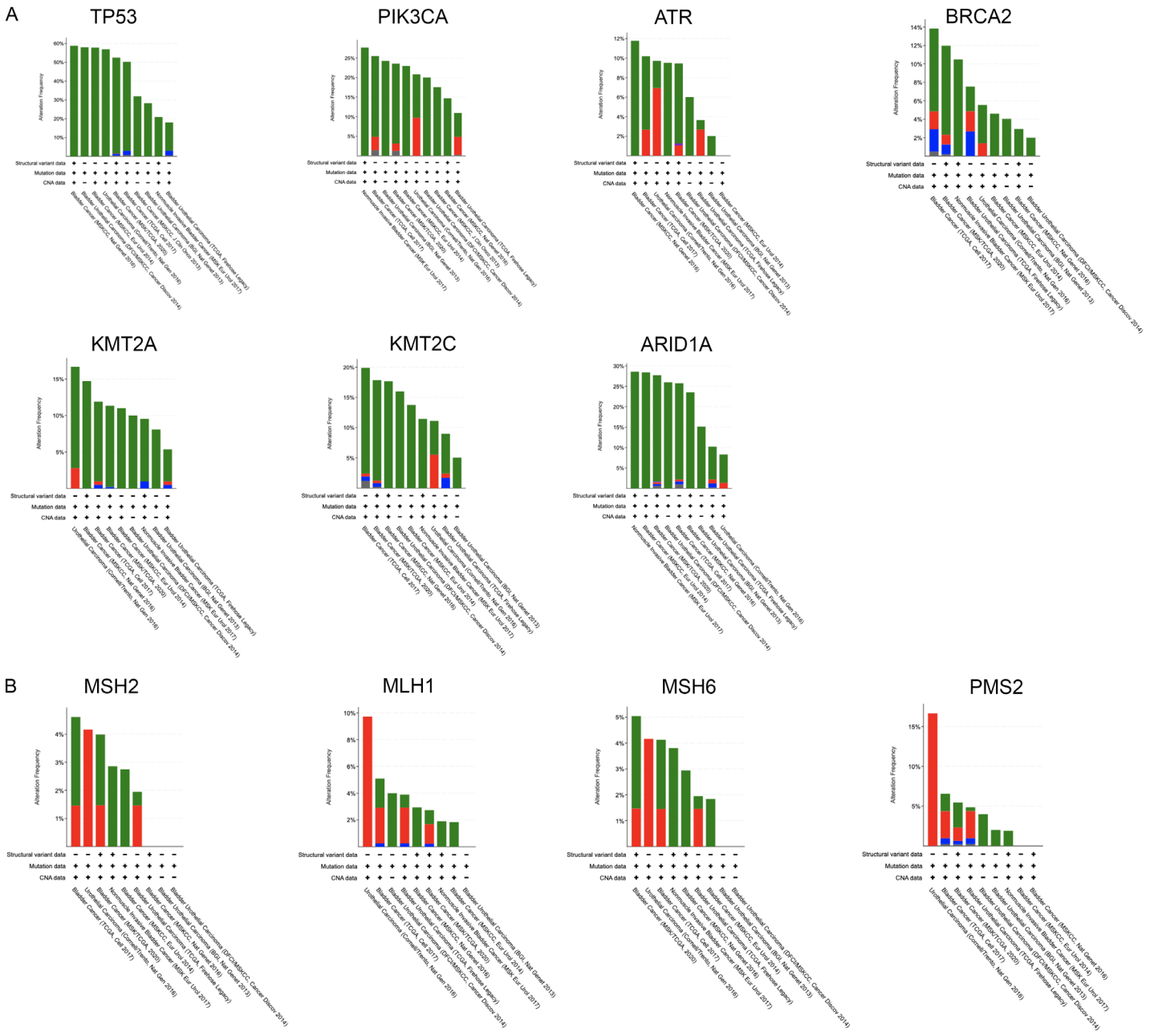
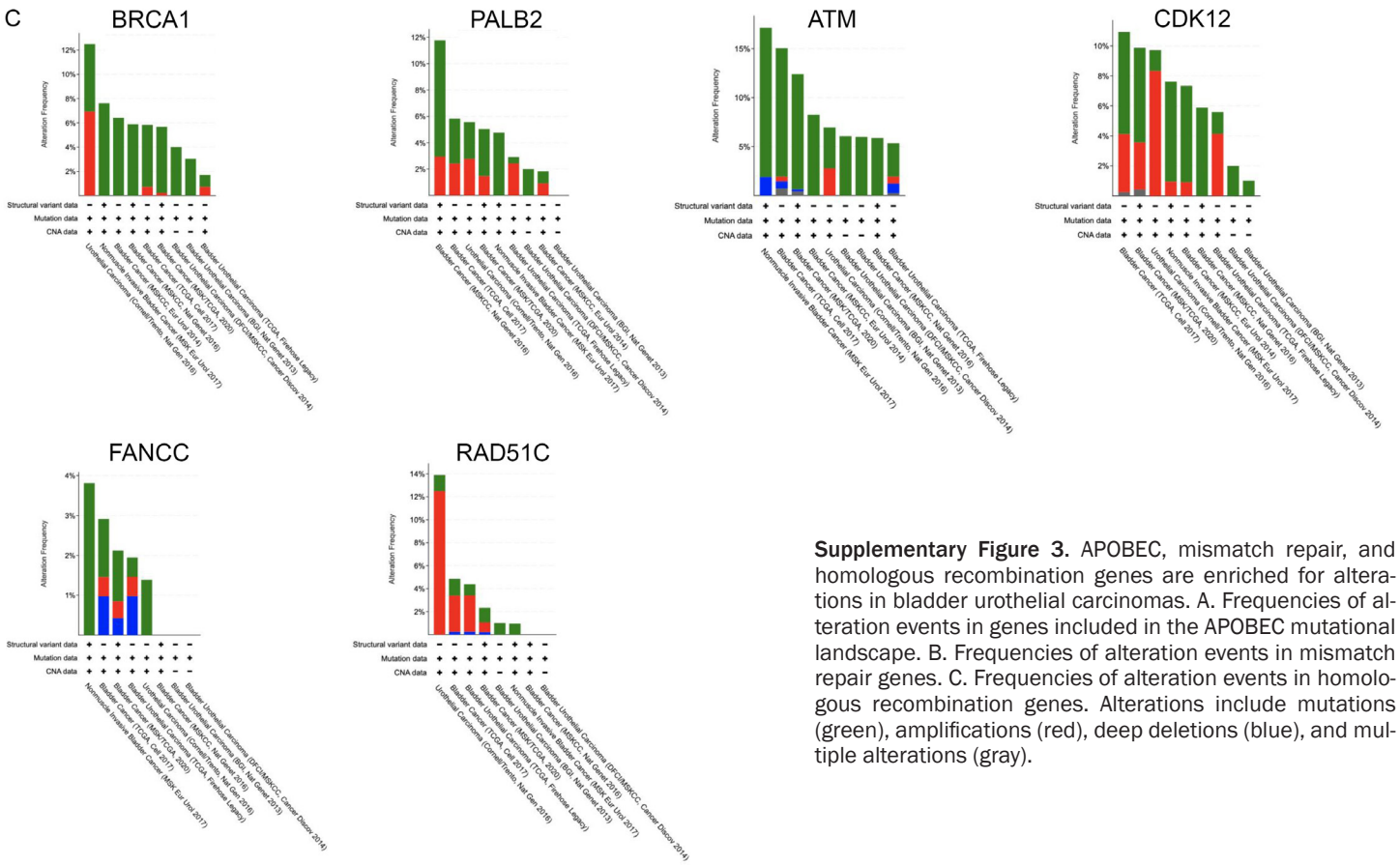


Supplementary Figure 1. CDKN1A mutations are prevalent in bladder urothelial carcinomas and predict a trend toward worse prognosis. A. CDKN1A alteration frequencies in all bladder urothelial carcinoma studies included in cBioPortal. Alterations include mutations (green), amplifications (red), deep deletions (blue), and multiple alterations (gray). B. Comparison of survival between patients with a CDKN1A alteration (red) and without (blue). Y-axis represents overall survival, which includes both progression-free and disease-free survival. X-axis represents overall survival in months. Logrank Test *P*-value = 0.138.

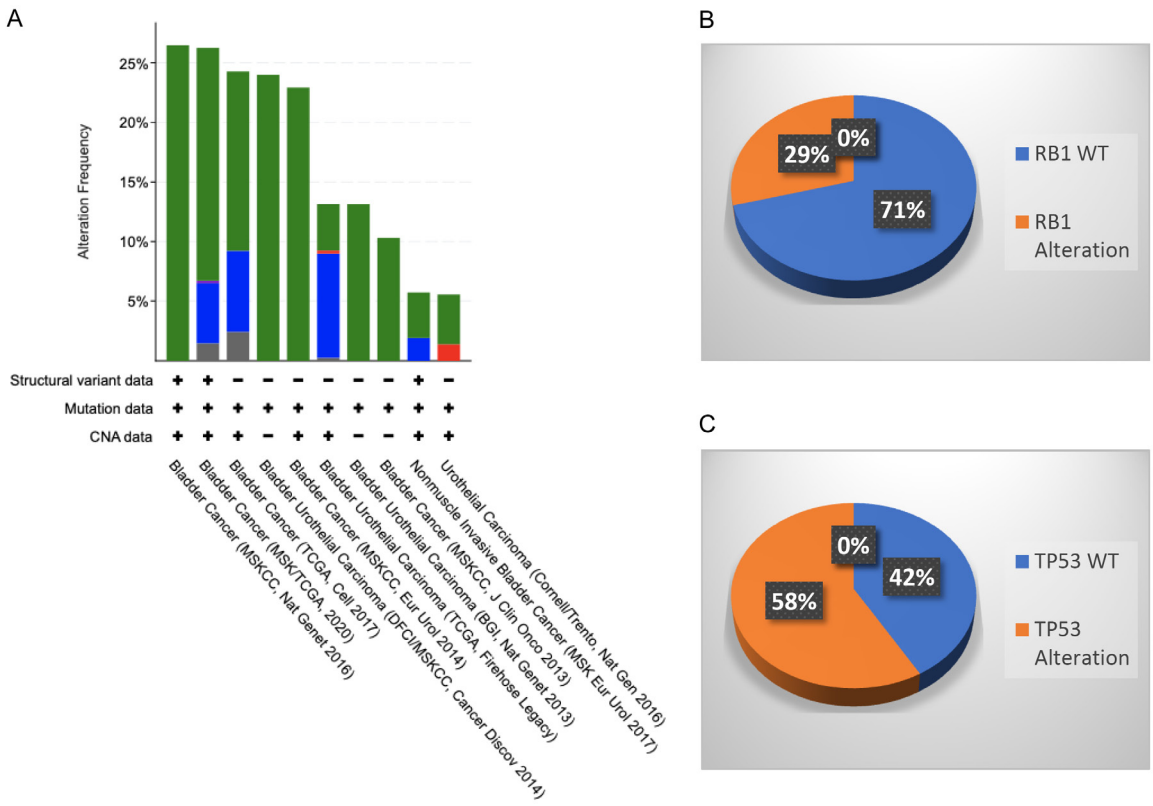


Supplementary Figure 2. TP53 truncating mutations are present across a number of cancer types, and TP53 missense mutations are most common in bladder urothelial carcinomas. A. Percentages of TP53 truncating mutations across the cancer types most strongly enriched for CDKN1A alterations in TCGA, in addition to lung cancers included in TCGA. B. Schematic of TP53 mutations. Depicted are driver missense mutations (dark green, 601 total), driver truncating mutations (black, 221 total), driver splice mutations (orange, 41 total), VUS missense mutations (light green, 9 total), driver in-frame mutations (maroon, 3 total), driver SV/fusion mutations (dark purple 1 total), VUS SV/fusion mutations (light purple, 1 total), and VUS in-frame mutations (brown, 1 total). C. All tumors with a CDKN1A alteration categorized by TP53 status.

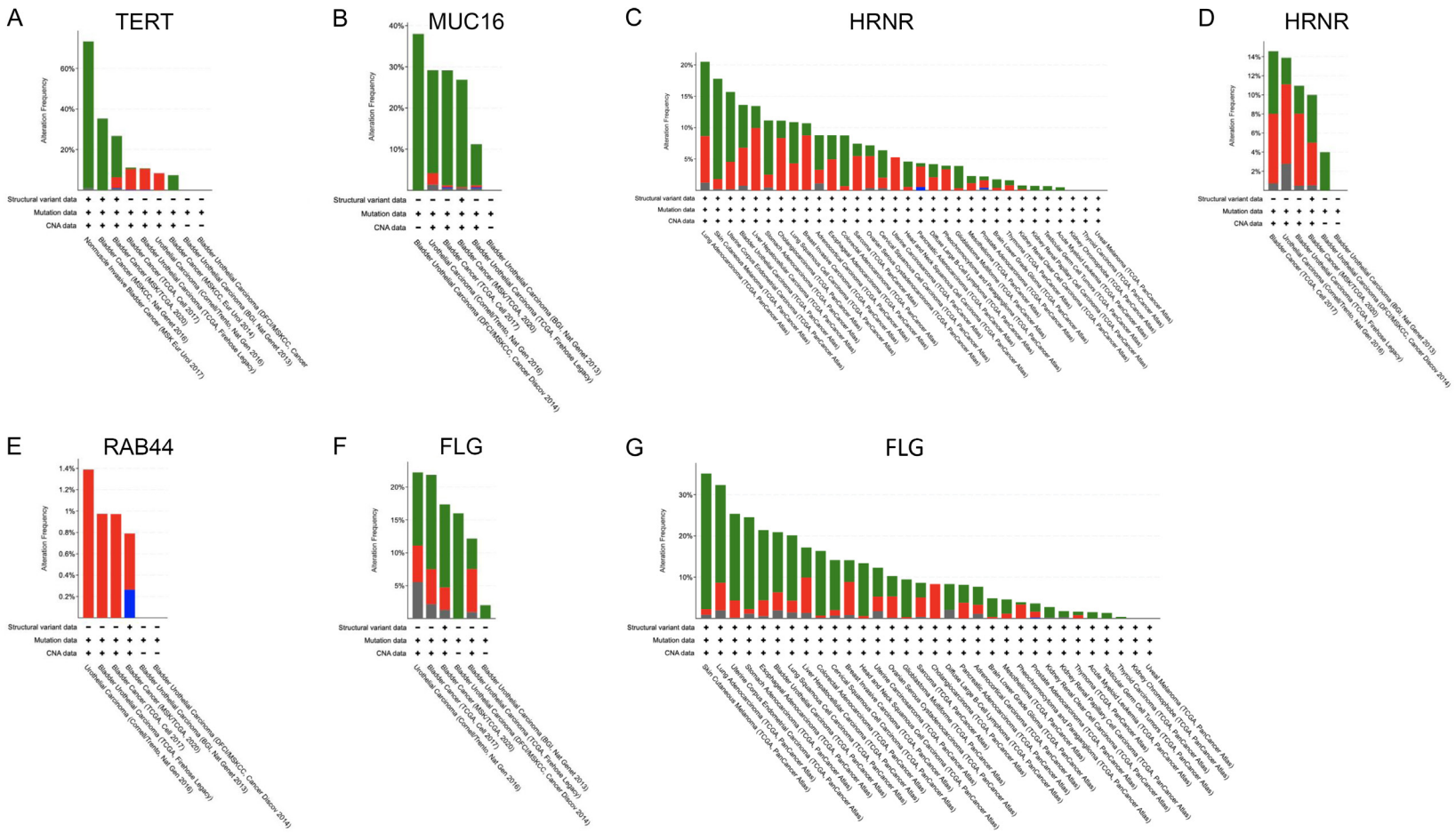

A
TP53
PIK3CA
ATR
BRCA2
KMT2A
KMT2C
ARID1A
B
MSH2
MLH1
MSH6
PMS2
Alteration Frequency
Structural variant data
Mutation data
CNA data



Supplementary Figure 3. APOBEC, mismatch repair, and homologous recombination genes are enriched for alterations in bladder urothelial carcinomas. A. Frequencies of alteration events in genes included in the APOBEC mutational landscape. B. Frequencies of alteration events in mismatch repair genes. C. Frequencies of alteration events in homologous recombination genes. Alterations include mutations (green), amplifications (red), deep deletions (blue), and multiple alterations (gray).



Supplementary Figure 4. RB1 alterations are enriched in bladder urothelial carcinomas. A. RB1 alteration frequencies across all bladder urothelial carcinoma studies included in cBioPortal. Alterations include mutations (green), amplifications (red), deep deletions (blue), structural variants (purple), and multiple alterations (gray). B. All tumors with a CDKN1A alteration categorized by RB1 status. C. All tumors with both a CDKN1A and an RB1 alteration categorized by TP53 status.



Supplementary Figure 5. TERT, MUC16, RAB44, HRNR, and FLG alterations are enriched in bladder urothelial carcinomas. A. TERT alteration frequencies across all bladder urothelial carcinoma studies included in cBioPortal. B. MUC16 alteration frequencies across all bladder urothelial carcinoma studies included in cBioPortal. C. HRNR alteration frequencies in TCGA PanCancer Atlas Studies. D. HRNR alteration frequencies across all bladder urothelial carcinoma studies included in cBioPortal. E. RAB44 alteration frequencies across all bladder urothelial carcinoma studies included in cBioPortal. F. FLG alteration frequencies across all bladder urothelial carcinoma studies included in cBioPortal. G. FLG alteration frequencies in TCGA PanCancer Atlas Studies. Alterations include mutations (green), amplifications (red), deep deletions (blue), and multiple alterations (gray).