BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Next-Generation Sequencing Reveals Potential Predictive Biomarkers and Targets of Therapy for Urothelial Carcinoma *in Situ* of the Urinary Bladder

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Bacillus Calmette-Guérin instillation after removal of the tumor is the first line of treatment for urothelial carcinoma *in situ* (CIS), the precursor lesion of most muscle-invasive bladder cancers. Bacillus Calmette-Guérin therapy fails in >50% of cases, and second-line radical cystectomy is associated with overtreatment and drastic lifestyle consequences. Given the need for alternative bladder-preserving therapies, we identified genomic alterations (GAs) in urothelial CIS having the potential to predict response to targeted therapies. Laser-capture microdissection was applied to isolate 30 samples (25 CIS and 5 muscle controls) from 26 fresh-frozen cystectomy specimens. Targeted next-generation sequencing of 31 genes was performed. The panel comprised genes frequently affected in muscle-invasive bladder cancer of nonpapillary origin, focusing on potentially actionable GAs described to predict response to approved targeted therapies or drugs that are in registered clinical trials. Of CIS patients, 92% harbored at least one potentially actionable GA, which was identified in *TP53*/cell cycle pathway-related genes (eg, *TP53* and *MDM2*) in 72%, genes encoding chromatin-modifying proteins (eg, *ARID1A* and *KDM6A*) in 68%, DNA damage repair genes (eg, *BRCA2* and *ATM*) in 60%, and phosphatidylinositol 3-kinase/mitogen-activated protein kinase pathway genes (eg, *ERBB2* and *FGFR1*) in 36% of the cases. These data might help guide the selection of targeted therapies to be investigated in future clinical CIS trials, and they may provide a basis for future mechanistic studies of urothelial CIS pathogenesis. (*Am J Pathol* 2020; 190: 323–332; <https://doi.org/10.1016/j.ajpath.2019.10.004>)

With an estimated number of 380,000 new cases and 150,000 deaths per year worldwide, bladder cancer is the most common malignancy of the urinary tract.¹ More than 90% of all bladder tumors diagnosed in Europe and North America are urothelial carcinomas.² Most bladder cancers present as non-muscle-invasive, low-grade papillary carcinomas, characterized by *FGFR3* gene alterations and an excellent prognosis (5-year survival of approximately 90%). These tumors infrequently progress to muscle-invasive disease but frequently recur. In contrast, muscle-invasive bladder cancer (MIBC) is associated with an unfavorable prognosis (5-year survival <50%) and a high risk of progression to metastasis.² Most MIBC cases arise via carcinoma *in situ* (CIS), a flat high-grade lesion associated with *TP53* mutations. CIS is found in approximately 10% of all

patients diagnosed with non-muscle-invasive bladder cancer (NMIBC).³ The fraction of primary (isolated) CIS is estimated to be 3% of all patients diagnosed with bladder cancer.⁴ It is estimated that, without any treatment, 40% to 60% of CIS patients develop muscle invasion within 5 years after diagnosis.⁵ Currently, therapy for bladder cancer, including CIS, is mainly based on non-specific treatments [ie, chemotherapy or bacillus Calmette-Guérin (BCG) immunotherapy in addition to surgery], depending on the

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risk estimation for the disease diagnosed.^{6,7} Recently, the approval of immune checkpoint inhibitors by the US Food and Drug Administration and the European Medicines Agency has opened new therapeutic options for patients with advanced bladder cancer.⁸ For urothelial bladder cancer, immune checkpoint inhibitor drugs have been approved for the treatment of patients with metastatic bladder cancer as second-line therapy in the post-platinum setting or for cisplatin-ineligible individuals in first line if patients exhibit a combined positive score >10%.⁹ Although new-generation immunotherapies have been shown to result in durable responses and seem to be relatively well tolerated, most patients do not respond because of primary or acquired resistance mechanisms.¹⁰ For CIS patients, intravesical BCG-based immunotherapy after transurethral resection of tumor cells remains the standard of care, as recommended by the European Association of Urology and the American Urological Association.^{6,11} However, it is estimated that BCG therapy fails in >50% of cases,⁵ caused by cessation of treatment because of toxicity or resistance to BCG immunotherapy.¹² Radical cystectomy is recommended as second-line treatment in case of BCG failure.^{6,11} Although early radical cystectomy is associated with an excellent tumor-specific survival,¹³ the rate of overtreatment is assumed to be up to 50%⁵ and radical cystectomy is a morbid surgery significantly impacting quality of life.¹⁴ Taken together, there is large need for alternative bladder-preserving therapies for urothelial CIS, reflected by the wide landscape of active clinical trials in high-grade NMIBC.¹⁴

In recent years, advances in next-generation sequencing (NGS) technologies have led to substantial progress in the molecular characterization of several diseases, including bladder cancer, and thereby the identification of potential biomarkers and candidate therapeutic targets.^{15–20} Although most of the work focused on advanced bladder cancer,^{15–17,20} fewer advances have been made regarding NMIBC.^{18,19} Especially the genomic alterations (GAs) underlying the preinvasive CIS, the precursor lesion of fatal MIBC, have been characterized insufficiently so far.²¹ In this study, laser-capture microdissection (LCM) and targeted NGS were combined to identify therapeutically actionable GAs [mutations and copy number variations (CNVs)] in a cohort of fresh-frozen CIS.

Materials and Methods

Patients and Sample Preparation

From 26 cryopreserved cystectomy specimens, 30 samples (25 urothelial CIS and 5 smooth muscle control specimens) were isolated by LCM. The 26 patients were treated by cystectomy at the University Hospital RWTH Aachen (Aachen, Germany) between 2005 and 2011 and were retrospectively included in the present study. The study was

conducted at the University Hospital RWTH Aachen in accordance with local institutional review board—approved protocols of the Medical Faculty of the RWTH Aachen University (approval number EK 291/16) and the principles expressed in the Declaration of Helsinki. The clinicopathologic parameters for the 26 cystectomy cases are summarized in [Table 1](#) and [Supplemental Table S1](#). The pathologic diagnosis of each urothelial CIS case as well as the absence of cancer cells in smooth muscle controls (distant from tumor areas) were confirmed on hematoxylin and eosin—stained slides by an experienced uropathologist (R.K.) before LCM-based isolation of cells. Moreover, great care has been taken to isolate mere CIS regions without any contact to invasive areas. [Figure 1](#) shows a hematoxylin and eosin—stained tissue section of an exemplary cryopreserved CIS sample that is part of our CIS cohort.

For LCM of CIS (and smooth muscle) cells from the cryoconserved cystectomy specimens, ethanol-fixed, methylene blue—stained tissue sections (10 μ m thick) were prepared on nuclease- and nucleic acid—free membrane slides (Zeiss, Oberkochen, Germany) to isolate an area of 6 to 10 $\times 10^6$ μ m² per case using the PALM MicroBeam IP 230VZ (Zeiss) and adhesive caps (Zeiss). Control hematoxylin and eosin slides were prepared after every 10 sections to ensure the histology on the sample slides. DNA was extracted using the QIAamp DNA Micro kit (Qiagen, Hilden, Germany) and was quantified by a Qubit fluorometric assay (Thermo Fisher Scientific, Waltham, MA). To assess the quality of isolated DNA, a real-time quantitative PCR—based method using the TruSeq FFPE DNA Library Prep QC kit (Illumina, San Diego, CA) was applied.

Library Preparation and Targeted NGS

Genomic target regions for NGS analysis were selected as follows: to identify common GAs (mutations and CNVs) potentially inherent in CIS, publicly available sequencing data for MIBC of nonpapillary origin of The Cancer Genome Atlas network,¹⁵ accessible via the cBio cancer genomics portal version 3.1.0,²² were used. Of these data, potentially druggable GAs in genes impacting the selection of targeted anticancer therapies on the market or that are in registered clinical trials were further studied ([Supplemental Table S2](#)). Libraries were generated applying the TruSeq Custom Amplicon Low Input kit (Illumina), according to the manufacturer's instructions. In brief, at least 10 ng of high-quality DNA and 20 ng of medium-quality DNA (as determined by TruSeq FFPE DNA Library Prep QC kit) were used for hybridizing the custom target gene oligonucleotide pools. To improve the validity of the sequencing data, a dual-strand sequencing protocol making use of two sets of custom target gene oligonucleotide pools (named CATA and CATB) was applied, enabling sequence analysis of both complementary DNA strands. Subsequent to hybridization and an extension-ligation step, target regions were amplified using unique index adapter combinations

Table 1 Clinicopathologic Data of the CIS Patients Analyzed in This Study

Sample ID	Cystectomy	Sex	Age, years	Tumor grade	pT	pN	pM	Pretreatment
CIS01	1	M	82	G3	pT3	1	0	No
CIS02	2	F	66	G3	pT4	0	NA	No
MU05	2	F	66	G3	pT4	0	NA	No
CIS03	3	M	73	G3	pT3a	0	NA	No
CIS04	4	M	43	G3	pT2	0	NA	No
CIS05	5	M	75	G3	pT1	0	NA	No
CIS06	6	M	69	G3	pT4	1	NA	No
CIS07	7	M	62	G3	pT3a	2	NA	No
CIS08	8	F	77	G3	pT3a	1	NA	No
CIS09	9	M	81	G3	pT3b	0	NA	No
CIS10	10	M	72	G3	pT2a	0	NA	Yes
CIS11	11	M	50	G3	pT2	0	1	No
CIS13	12	F	70	G3	pT3a	1	NA	No
CIS14	13	M	77	G3	pT2	0	NA	Yes
CIS15	14	F	70	G3	pT2a	NA	NA	Yes
CIS16	15	M	66	G3	pT3b	0	NA	No
CIS17	16	M	76	G3	pT3a	0	NA	No
CIS18	17	M	76	G3	pT3a	0	NA	No
MU04	18	F	62	G3	pT2	0	NA	No
CIS20	19	M	79	G3	pT3	2	NA	No
CIS21	20	F	75	G3	pT2a	0	NA	No
MU01	20	F	75	G3	pT2a	0	NA	No
CIS22	21	M	61	G2 high grade	pT1b	0	NA	No
CIS23	22	M	57	G3	pTis	0	NA	No
MU03	22	M	57	G3	pTis	0	NA	No
CIS24	23	M	63	G2 high grade	pT1	0	NA	No
CIS25	24	M	69	G3	pT3a	NA	NA	Yes
CIS26	25	M	65	G3	pT1a	0	NA	Yes
MU02	25	M	65	G3	pT1a	0	NA	Yes
CIS27	26	M	60	G3	pT4	1	NA	No

CIS, carcinoma *in situ*; F, female; ID, identification; M, male; MU, smooth muscle sample; NA, not available; pM, distant metastasis status; pN, regional lymph node status; pT, primary tumor classification.

(Illumina). After cleanup, library quality control was assessed by bioanalyzer analysis (Agilent Technologies, Santa Clara, CA). Finally, normalized libraries were sequenced on a NextSeq 500 platform (Illumina) using a NextSeq 500/550 Mid Output kit (2 × 150 cycles; Illumina). For single-nucleotide variant analysis, bam-file generation was performed with the DNA amplicon module version 2.0.0 (Basespace; Illumina). Single-nucleotide variant analysis was conducted using Sequence Pilot Software version 4.4 (SeqNext module; JSI Medical Systems, Ettenheim, Germany). For all analyzed samples, region of interest coverage was >96% at 300×. Missense and silent variants with an allele frequency >2% in the normal population [according to 1000 Genomes (<http://www.internationalgenome.org>, last accessed September 2, 2019) or dbSNP version 153 (<https://www.ncbi.nlm.nih.gov/snp>, last accessed September 27, 2019)] and non-splicing—relevant silent and intronic variants in general were considered nonpathogenic. In addition, all untranslated region and missense variants annotated as benign or likely benign in the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar>, last accessed September 27,

2019) were also excluded. The *TERT* gene promoter was checked for the two known recurrent mutations: c.-124C>T and c.-146C>T. The cutoff for the variant allele frequency was set to 5%. As reference genome for panel design, hg19 was used, whereas hg38 served as reference genome for mutation annotation. For CNV analysis, a recently developed in-house algorithm (validated using three NGS panels; *n* = 144 samples), based on an exponential growth model for amplification of PCR products, was applied. For every gene analyzed for CNVs, at least 10 amplicons were included in the panel design. Coverage data were normalized; and amplicon clustering, based on the PCR efficiency among all samples, was performed (for each amplicon). Genes were considered harboring a CNV if at least 30% of the amplicons of a gene were recognized as outliers in most of the (five) response models built within a cluster. The underlying reference genome for the panel design was hg19.

The raw sequencing data of this study are deposited in the National Center for Biotechnology Information Sequence Read Archive version 2.15.0 (<https://www.ncbi.nlm.nih.gov/sra>; accession number PRJNA551281).

Figure 1 Hematoxylin and eosin–stained tissue section of an exemplary cryopreserved carcinoma *in situ* (CIS) sample used for laser-capture microdissection. **A:** Overview of a specimen with maintained luminal urothelium, edematous stroma, and lymphofollicular infiltrates. No invasive tumor is seen in proximity, and detrusor muscle is present. The **boxed area** is shown at higher magnification in **B**. **B:** CIS of the urothelium, best maintained in a fold but also in adjacent flat urothelium. Accompanying mild chronic edematous cystitis in the adjacent stroma. Scale bars: 5 mm (**A**); 250 μ m (**B**).

Fluorescence *in Situ* Hybridization

To validate NGS-based detection of *MDM2* gene amplification, fluorescence *in situ* hybridization was performed on corresponding formalin-fixed, paraffin-embedded tissue samples using the ZytoLight SPEC *MDM2/CEN 12* Dual Color Probe (ZytoVision GmbH, Bremerhaven, Germany). The test is based on the use of two fluorescently labeled oligonucleotide probes: a *MDM2*-specific probe (green fluorochrome ZyGreen) and a probe targeting the centromeric region of chromosome 12 (orange fluorochrome ZyOrange) on which the *MDM2* locus is located. The test was performed according to the manufacturer's instructions. In brief, deparaffinized and rehydrated tissue sections were immersed in pretreatment buffer for 20 minutes at 98°C, followed by enzymatic pepsin digestion for 10 minutes at 37°C. Subsequently, the dual-color probe was applied to the tissue sections, followed by DNA denaturation at 75°C and a hybridization step at 37°C overnight. After stringent washing and DAPI counterstaining, hybridized probes were visualized by fluorescence microscopy (Axiovert S135 microscope; Zeiss). A minimum of 50 nuclei per case was analyzed, and the mean ratio of green (*MDM2*) over orange (*CEN 12*) hybridization signals was calculated. A case showing a ratio of >2.0 was considered *MDM2*-amplified.

Results

The NGS tissue cohort comprised 25 fresh-frozen urothelial CIS samples from 25 cystectomy specimens of 5 female and 20 male patients with a mean age of 69 years (range, 43 to 82 years). For control purposes, five normal smooth muscle samples were also included in the cohort. Tumor multifocality was observed in 96% (24/25) of cystectomies, and 80% (20/25) of patients exhibited muscle-invasive disease. Of the patients, 80% (20/25) did not receive pretreatment before cystectomy (Table 1 and Supplemental Table S1).

Mutations and CNVs in 31 genes (Supplemental Table S2) were analyzed. In total, 108 GAs (mutations and CNVs), including multiple aberrations per gene, were identified in 25 LCM-isolated CIS samples, with an average of 4.3 alterations per sample (range, 0 to 13) (Supplemental Tables S3 and S4). Affected genes per CIS case are summarized in Figure 2. With 52% (13/25) of affected cases, *TERT* gene promoter mutations were the most frequent GAs detected (Figure 2). Of CIS specimens, 72% (18/25) harbored at least one GA in genes of the *TP53*/cell cycle pathway (*TP53*, *MDM2*, *CCND1*, *CDKN1A*, *CDKN2A*, *CCNE1*, and *FBXW7*). Genes encoding chromatin-modifying proteins (*ARID1A*, *KDM6A*, *CREBBP*, and *EP300*) were impacted with a similar frequency of 68% (17/25) of cases. Moreover, DNA damage response genes (*BRCA2*, *ATM*, *BRCA1*, and *ERCC2*) exhibited variants in 60% (15/25) and phosphatidylinositol 3-kinase/mitogen-activated protein kinase pathway genes (*ERBB2*, *FGFR1*, *PIK3CA*, *EGFR*, *RAF1*, and *NF1*) exhibited variants in 36% (9/25) of CIS patients. The validity of the CNV data was approved in fluorescence *in situ* hybridization experiments exemplarily performed for *MDM2* (Supplemental Figure S1).

Of all GAs (mutations and CNVs), 86% (93/108) were considered potentially actionable, including variants of yet unknown significance in genes with predictive potential (Supplemental Tables S3 and S4). On average, 3.7 potentially actionable GAs were detected per patient (range, 0 to 11); and 92% (23/25) of CIS cases harbored at least one GA in genes potentially impacting the selection of targeted therapies. Frequently altered genes found in CIS that were considered potentially actionable and potential related therapeutic options are summarized in Figure 3 and are listed in detail in Supplemental Table S5.^{23–66}

Discussion

In contrast to MIBC, the molecular characterization of NMIBC lacks behind.^{21,67} Although the number of studies addressing this lack of knowledge is increasing,^{18,19,68–70} most work studying NMIBC by NGS spared aggressive CIS lesions, probably because of difficulties related to limited availability of tissue material.^{21,71} To the best of our knowledge, the present work studied the currently largest cohort of urothelial CIS samples of the urinary bladder using an NGS-based approach to identify GAs with the

Figure 2 Mutations and copy number variations identified in a cohort of 25 laser-capture microdissected, fresh-frozen urothelial carcinoma *in situ* (CIS) samples. Analyzed genes without detected alterations are not shown.

potential to guide the investigation of targeted therapies in future clinical trials.

With a frequency of 52%, *TERT* gene promoter mutations were the most common genetic alterations in our CIS cohort; that is comparable to mutational rates in CIS observed in earlier studies.^{19,72} *TERT* promoter mutations detected in the present study were confined to two hot spot positions recently described in bladder cancer⁷³ (namely, to the positions -124 and -146 bp upstream of the ATG

translation start codon in 77% and 23% of *TERT*-mutated cases, respectively).

In accordance with the proposed model of a distinct development of papillary and flat urothelial lesions,² CIS cases in our study were characterized by frequent *TP53* mutations (44%), but complete absence of activating *FGFR3* alterations. Deletions in the *CDKN2A* gene locus were detected at obviously lower frequency in our cohort (4%) compared with MIBC cases of nonpapillary origin (31%),²⁰ which is in accordance with observations made in two recent NMIBC studies.^{19,69} The observed discrepancy might be attributable to a lower sensitivity of our amplicon-based CNV detection method compared with the array-based technology applied in the MIBC The Cancer Genome Atlas study²⁰ or might be related to CIS biology.

In addition to frequent aberrations in the *TP53*/cell cycle pathway (72%), genes encoding chromatin-modifying proteins (*ARID1A*, *KDM6A*, *CREBBP*, and *EP300*) were found to be altered at a similar rate (68%), followed by aberrations in DNA damage repair-associated genes (*BRCA2*, *ATM*, *BRCA1*, and *ERCC2*) in 60% of the analyzed CIS samples. A high prevalence of alterations in chromatin-modifying and DNA damage pathway genes in high-grade NMIBC¹⁹ and MIBC²⁰ has been noted before.

More important, 92% of CIS patients harbored at least one GA potentially predicting response to targeted therapy. Of these, *TP53* was the most frequently mutated gene analyzed in this study. Although p53 itself is not a therapeutic target, recent data suggest that *TP53*-mutated cancer cells, showing a defective G₁ checkpoint, rely on an intact G₂-M cell cycle checkpoint to ensure cell cycle arrest and repair in case of DNA damage.²⁴ Inhibition of either of the two kinases WEE1 G₂ checkpoint kinase (WEE1) or checkpoint kinase 1 (CHEK1), regulating G₂-M checkpoint activation, resulted in synthetic lethality in *TP53*-mutant cells. More important, combination of a WEE1/CHEK1 inhibitor with a DNA-damaging drug enhanced cancer cell death.²⁴ In bladder cancer, a similar reliance on an intact G₂-M checkpoint was noted, especially in *TP53*/*CDKN1A* double-mutant cells.²³ WEE1/CHEK1 inhibitors are currently investigated in clinical trials in solid tumors showing cell cycle/DNA repair defects (including *TP53* mutations) as monotherapy or in combination with DNA-damage inducing drugs, such as US Food and Drug Administration—approved poly (ADP-ribose) polymerase (PARP) inhibitors (<https://clinicaltrials.gov>; NCT02797964 and NCT03330847).

Alterations in *ARID1A* have been observed in 36% of CIS cases, comprising a high portion of samples harboring inactivating truncating mutations and gene deletions (78%). AT-rich interaction domain 1A (*ARID1A*) is a component of switch/sucrose non-fermentable chromatin remodeling complexes, potential tumor suppressors that are mutated in approximately 20% across human cancers.⁷⁴ Inactivation of switch/sucrose non-fermentable complexes, including

Figure 3 Potentially actionable genomic carcinoma *in situ* (CIS) alterations and suggested targeted drugs. **Top:** Impacted pathways and frequency of CIS cases harboring at least one potentially actionable genomic alteration in the respective pathway. The three most frequently affected genes of each pathway are depicted. **Bottom:** Potential targeted therapies. CDK4/6i, cyclin dependent kinase 4/6 inhibitor; CHEKi, checkpoint kinase inhibitor; CREBBPi, CREB binding protein inhibitor; DDR, DNA damage response; EP300, E1A binding protein p300; ERBB2i, erb-b2 receptor tyrosine kinase 2; EZH2i, enhancer of zeste 2 polycomb repressive complex 2 subunit inhibitor; FGFRi, fibroblast growth factor receptor inhibitor; MAPK, mitogen-activated protein kinase; MDM2i, MDM2 proto-oncogene inhibitor; PARPi, poly (ADP-ribose) polymerase inhibitor; PI3K, phosphatidylinositol 3-kinase; PI3Ki, phosphatidylinositol 3-kinase inhibitor.

ARID1A deficiency, has been proposed to be a predictive biomarker for a plethora of targeted therapies in cancer.^{25–27,29} For example, it has been observed that ARID1A loss of function disabled cell cycle checkpoint activation and thus proper DNA repair, sensitizing cells to PARP inhibitors.²⁵ Other data suggested that ARID1A deficiency might impair DNA topoisomerase II alpha function, resulting in an accumulation of DNA lesions and thus enhanced reliance on ATR serine/threonine kinase activity for checkpoint activation and repair.^{26,75} The authors demonstrated that application of ATR serine/threonine kinase inhibitors, currently tested in clinical trials in advanced bladder cancer and other solid tumors (<https://clinicaltrials.gov>; NCT03682289 and NCT02567409), might be a rational therapy for ARID1A-deficient carcinomas.²⁶

In addition to *ARID1A* alterations potentially enhancing response to PARP inhibitors, 56% of CIS samples in this study exhibited at least one GA in the DNA damage repair genes *BRCA1*, *BRCA2*, and *ATM*, including pathogenic missense variants, gene deletions, and missense mutations of unknown significance. Inactivation of homologous recombination—associated genes is known to sensitize cancer cells to PARP inhibition.³² The potential benefit of PARP inhibitors is currently studied in patients with advanced bladder cancer (<https://clinicaltrials.gov>; NCT03375307 and

NCT03397394). Moreover, alterations in DNA damage response and repair-related genes, including *BRCA1*, *BRCA2*, *ATM*, and *ERCC2* (impacted in 60% of samples analyzed in this study), have been associated with improved response to platinum chemotherapy in advanced urothelial carcinoma.³¹ Cisplatin-based combination therapies, currently studied in high-risk NMIBC (<https://clinicaltrials.gov>; NCT02202772), might represent a bladder-preserving treatment option for a subgroup of CIS patients.

Other genes encoding chromatin-modifying proteins have been found to be frequently altered in our CIS cohort (namely, *KDM6A* in 24% of cases as well as *CREBBP* and *EP300* in 20% of samples each). Concerning *KDM6A*, encoding a histone lysine demethylase,⁷⁶ most altered cases (83%) harbored inactivating truncating mutations, as noted recently in NMIBC.^{19,68} More important, preclinical data showed that lysine demethylase 6A deficiency sensitized bladder cancer cells to inhibition of enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), antagonizing lysine demethylase 6A activity³⁴; and a scheduled clinical trial (<https://clinicaltrials.gov>; NCT03854474) in advanced bladder cancer now aims to study the potential benefit of combined pembrolizumab and tazemetostat (EZH2 inhibitor) therapy. Inactivating truncating mutations or gene deletions in either *CREBBP* or *EP300*,

encoding two homologous histone acetyltransferases,⁷⁷ were detected in 20% of CIS cases in this study. Although recent clinical data do not support the use of histone deacetylase inhibitors for treatment of *CREBBP/EP300*-mutated bladder cancers,³⁶ a synthetic lethal relationship between E1A binding protein p300 (EP300) and CREB binding protein (CREBBP) has been described in cancer cells³⁷ and might have potential for bladder cancer therapy as EP300/CREBBP bromodomain inhibitors have recently entered clinical trials (<https://clinicaltrials.gov>; NCT03568656).

Another important finding of this study is the presence of alterations in known therapeutic drug target genes of the phosphatidylinositol 3-kinase/mitogen-activated protein kinase pathway [namely, *ERBB2* (20%), *FGFR1* and *PIK3CA* (both 12%), and *EGFR* and *RAF1* (both 4%)]. The *ERBB2* locus was altered by amplification (4%) and missense mutations in the extracellular domain (16%), including the pathogenic activating S310F mutation⁷⁸ and a variant of unknown significance in two cases each. *ERBB2* amplification is a relatively rare event in CIS,³⁸ and variants impacting amino acid position S310 (S310F/Y) are the most frequent missense mutations found in *ERBB2* in bladder cancer.²⁰ Although *ERBB2*-targeted therapies are US Food and Drug Administration–approved for *ERBB2*-positive breast and gastric cancer,⁷⁹ results of previous clinical trials in advanced bladder, using drugs inhibiting *ERBB2* signaling (trastuzumab and lapatinib), are inconsistent.^{80–82} More important, we have recently demonstrated moderate to strong positivity for *ERBB2* in 65% of CIS samples and only weak expression in adjacent normal urothelium.³⁸ Thus, as the driver status of *ERBB2* in bladder cancer is unclear,⁸³ antibody-drug conjugates, such as trastuzumab emtansine (T-DM1), are suggested as a rational therapy for urothelial CIS, supported by preclinical data in bladder cancer.³⁹ Clinical trials in late-stage bladder cancer investigating T-DM1 are underway, but results are not available yet (<https://clinicaltrials.gov>; eg, NCT02999672).

Although this work comprises the currently largest cohort of urothelial CIS samples analyzed by an NGS-based approach, it is limited to carefully mapped frozen cystectomy samples to find unequivocal and sufficient material for analysis. Larger-scale studies are needed to validate these findings and may partially be possible by targeted analysis of formalin-fixed transurethral resection samples, for which we have an extended data base.³⁸ Also, this sequencing study was focused on a specific gene set; and it is meaningful to perform more comprehensive analyses of this entity in upcoming studies. More important, because of our primary motive of finding therapy targets, matched control samples were not analyzed for each CIS case and a certain discrimination of germline and somatic variants was thus not possible.

In summary, targeted NGS of fresh-frozen urothelial CIS samples identified at least one potentially actionable alteration in most CIS cases. These data should help in guiding

the selection of targeted intravesical/systemic therapies that should be investigated in future clinical trials.

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Author Contributions

S.G. and R.K. conceptualized the study; S.G., N.O.-B., U.S., and K.G. designed the methods; S.G., U.S., and I.L. performed the experiments; N.O.-B., K.G., and S.G. analyzed the results; K.G. and N.O.-B. developed the in-house software for CNV analysis; S.G. wrote the manuscript; and E.L., N.O.-B., R.K., N.T.G., I.L., A.H., K.G., U.S., and K.L.-D. edited the manuscript; N.T.G., K.L.-D., and A.H. provided resources; S.G. and R.K. supervised the study; S.G. acquired funding.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2019.10.004>.

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