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Original article

KDM6A expression loss is frequent in low grade non-invasive urothelial carcinomas of the urinary bladder

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Summary

Objective. The gene lysine demethylase 6A (*KDM6A*) located on chromosome Xp11 often shows truncating mutations in urothelial carcinoma. Mutations resulting in protein expression loss can be detected by immunohistochemistry (IHC).

Methods. A tissue microarray with >2,500 bladder tumors was analyzed by IHC. 78 cancers were sequenced for KDM6A.

Results. KDM6A expression loss decreased from 36% of 345 pTaG2 low-grade to 23% of 152 pTaG2 high-grade and 18.5% of 92 pTaG3 tumors (p=0.0004) but not further in pT2-4 cancers (17.2-21.9%). KDM6A staining was unrelated to pT, pN, grade, and overall survival (p>0.1894) in 636 patients with pT2-4 cancers. KDM6A loss was more common in male (22.2%) than in female patients (15.4%; p=0.0067), and in tumors from males with Y-chromosome loss (36.1%) than without Y-loss (16.3%; p<0.0001). A KDM6A loss occurred in all 15 male and in 17 (74%) of 23 female patients with a truncating KDM6A mutation, but only 15 (75%) of 20 male and 17 (81%) of 21 female patients with KDM6A expression loss had a truncating mutation.

Conclusions. KDM6A expression loss is frequent in urothelial carcinoma and mostly due to truncating mutations. KDM6A IHC may be a useful tool for the distinction of neoplastic from non-neoplastic urothelial cells in follow-up examinations of patients with KDM6A deficient cancers.

Key words: KDM6A deficiency, *KDM6A* mutation, urothelial carcinoma, immunohistochemistry, tissue microarray

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Introduction

Carcinoma of the urinary bladder is one of the most common malignant tumor types worldwide. About 80% of patients present with non-invasive (pTa) or minimally invasive (pT1) cancers which can be removed by transurethral resection. Most patients have a good prognosis, but at least 20% of pT1 cancers will further progress to muscle-invasive disease. At this

stage, radical cystectomy (RC) is the surgical standard of care although distant metastasis develops in up to 50% of patients, most of whom eventually die from their disease. Significant shortcomings for early detection and appropriate individual treatment of bladder cancer include a low sensitivity of urine cytology [1], limitations in the assessment of cancer aggressiveness [2], and the lack of efficient treatment in case of systemic disease [3]. It is hoped that our increased understanding of the molecular changes underlying bladder cancer development and progression will eventually lead to improved patient management.

Inactivating mutations of lysine demethylase 6A (*KDM6A*) belong to the most commonly found molecular aberrations in urothelial carcinomas, occurring in 30-52% of pTa and in 23-25% of pT2-4 cancers [4,5]. *KDM6A*, also known as UTX (ubiquitously transcribed X chromosome tetratricopeptide repeat protein) is an epigenetic regulator that represents a critical part of the COMPASS-like complex. This protein complex can demethylate histone H3 at its 27th amino acid (H3K27me2/3) and thus defines a repressive histone modification that leads to transcriptional inactivation of various genes. Both, tumor suppressive and oncogenic roles of *KDM6A* have been proposed in cancer [6]. In a study on bladder cancer cell lines, KDM6A loss resulted in a dependency on enhancer of zeste homolog 2 (EZH2), a potential therapeutic target, and EZH2 inhibition resulted in decreased growth of *KDM6A*-null patient-derived cell line xenografts [7]. Large scale studies on the role of KDM6A protein expression in bladder cancer are so far lacking. However, because of the fact that most *KDM6A* mutations are truncating, that *KDM6A* localizes to chromosome Xp11.3, and that due to imprinting not only males but also females should have only one active copy of the *KDM6A* gene, many *KDM6A* mutations must result in a complete loss of KDM6A protein expression and become detectable by immunohistochemistry (IHC).

To study the prevalence and the potential diagnostic and prognostic role of KDM6A expression loss, a previously collected cohort of more than 2,700 urothelial bladder carcinomas was analyzed by IHC in a tissue microarray (TMA) format, and the results were compared with histopathological parameters of cancer aggressiveness, patient outcome, *KDM6A* sequencing data, and previously collected data on the Y chromosome status of our cohort.

Materials and methods

Tissue Microarrays (TMAs)

Our set of TMAs contained one sample each from 2,710 urothelial tumors of the bladder archived at the Institute of Pathology, University Hospital Hamburg, Germany, Institute of Pathology, Charité Berlin, Germany, Department of Pathology, Academic Hospital Fuerth, Germany, or Department of Pathology, Helios Hospital Bad Saarow, Germany, and/or treated at Department of Urology, University Hospital Hamburg, Germany, Department of Urology, Charité Berlin, Germany, Department of Urology, Helios Hospital Bad Saarow, Germany, Department of Urology, Albertinen Hospital, Hamburg, Germany, and Department of Urology and Urological Oncology, Pomeranian Medical University, Szczecin, Poland. Patients at each center were treated according to the guidelines at the time. In brief, patients with pTa/pT1 disease underwent a transurethral resection of the bladder tumor with or without adjuvant instillation therapy, while most patients with pT2-4 disease were treated by radical cystectomy. Available histopathological data including grade, tumor stage (pT), lymph node status (pN), and status of blood vessel (V) and lymphatic vessel (L) infiltration are shown in Table I. The grading of pTa tumors includ-

Table I. Study cohort.

	Study cohort on TMA (n=2710)
Follow-up	636
Months	
Mean	26.7
Median	15.0
Pathological tumor stage	
pTa	887 (38.4%)
pT1	49 (2.1%)
pT2	462 (20.0%)
pT3	615 (26.6%)
pT4	298 (12.9%)
Tumor grade	
G1	7 (0.3%)
G2	812 (30.3%)
G3	1858 (69.4%)
Pathological lymph node status	
pN0	734 (62.0%)
pN+	449 (38.0%)
Resection margin status	
R0	595 (80.6%)
R1	143 (19.4%)
Lymphatic vessel infiltration	
L0	275 (49.5%)
L1	281 (50.5%)
Blood vessel infiltration	
V0	450 (74.4%)
V1	155 (25.6%)

Percent in the column "study cohort on TMA" refers to the fraction of samples across each category. Numbers do not always add up to 2,710 in the different categories because of cases with missing data.

ed both a classification according to WHO 2004 which were valid at the time of the respective diagnoses. A centralized review of the cases was not done. Clinical follow-up data (overall survival; OS) were available from 636 patients with pT2-4 carcinomas treated by cystectomy. The tissues were fixed in 4% buffered formalin and then embedded in paraffin. For TMA manufacturing, one tissue spot (diameter: 0.6 mm) was transmitted from a cancer containing donor block into an empty recipient paraffin block. Conventional whole sections were prepared from donor tissue blocks of all 6 cancers from females with truncating *KDM6A* mutations but retained KDM6A staining in the TMA spots. Data on Y-chromosome loss was expanded from an earlier study [8]. The use of archived remnants of diagnostic tissues for TMA manufacturing, their analysis for research purposes, and patient data were according to local laws (HmbKHG, §12) and the analysis was approved by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work was carried out in compliance with the Helsinki Declaration.

Immunohistochemistry (IHC)

Freshly prepared TMA sections were immunostained on one day in one experiment. Slides were deparaffinized with xylol, rehydrated through a graded alcohol series and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at 121°C in pH 7.8 target retrieval solution (Agilent, CA, USA; #S2367). Endogenous peroxidase activity was blocked with peroxidase blocking solution (Agilent, CA, USA; #52023) for 10 min. Primary antibody specific for KDM6A (rabbit recombinant monoclonal, clone HMV-311, cat. # 2082-267M, ardoci GmbH, Hamburg, Germany) was applied at 37°C for 60 min at a dilution of 1:150. Primary antibody specific for Ki-67 (rabbit recombinant monoclonal, clone MSVA-267M, cat. # 2843-U3223, MS Validated antibodies GmbH, Hamburg, Germany) was applied at 37°C for 60 min at a dilution of 1:150. Bound antibody was then visualized using the EnVision kit (Agilent, CA, USA; #K5007) according to the manufacturer's directions. The sections were counterstained with haemalaun. For KDM6A, the average staining intensity of unequivocally neoplastic cells was estimated as 0, 1+, 2+, 3+. For the classification of a tumor as negative (0), presence of unequivocal KDM6A staining in tumor adjacent normal cells was required. Tumors with complete absence of KDM6A staining in cancer cells but a lack of stromal cells with unequivocal KDM6A positivity were considered "non-informative". For Ki-67, the proportion of tumor cells with nuclear staining was recorded.

KDM6A sequencing

A total of 20 pTa tumors from males with a KDM6A expression loss, 21 pTa-pT4 tumors from females with a KDM6A expression loss and 37 pTa-pT4 tumors (16 male, 21 female) with retained KDM6A expression were subjected to sequencing. DNA was extracted from FFPE specimens using the Maxwell® RSC DNA FFPE Kit (Promega Corporation, Madison, WI, USA) and the Maxwell RSC 48 Instrument according to the manufacturer's instructions. Targeted gene sequencing was performed on a MiniSeq (Illumina, San Diego, CA, USA) platform with a custom primer panel that covered all exons of the *KDM6A* gene (for details see Supplementary Tab. I). The DNA libraries were prepared using the AmpliSeq Library PLUS kit and Illumina CD indexes set B (Illumina, San Diego, CA, USA). Sample preparation was carried out in a PCR-based workflow following Illumina's AmpliSeq protocol. In brief, 100-200 ng DNA were used in the PCR reaction as follows. Step 1: 1x at 99°C for 2 min, step 2: 20x at 99°C for 15 sec followed by 60°C for 4 min, step 3: 1x at 10°C hold) followed by partial digestion of the amplicons, ligation of indexes, clean up, amplification of the library, and second clean up. Libraries were sequenced on an Illumina MiniSeq according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The sequencing data were analyzed using the CLC software package CLC Genomics Workbench v.22.0.2 (Qiagen, Aarhus A/S).

Statistics

Statistical calculations were performed with JMP 16 software (SAS Institute Inc., NC, USA). Contingency tables and the chi²-test were performed to search for associations between KDM6A expression loss, tumor phenotype, and molecular parameters. Survival curves were calculated according to Kaplan-Meier. The Log-Rank test was applied to detect significant differences between groups.

Results

Technical issues

Of 2,710 urothelial cancer samples, 2,125 (78.4%) were interpretable for KDM6A IHC. Reasons for non-informative cases included absence of KDM6A positive stroma cells or of unequivocal cancer cells in the TMA spot, as well a complete lack of tissue spots.

KDM6A immunostaining

A distinct nuclear KDM6A staining was always seen in normal urothelium although it tended to be less intense in umbrella cells. KDM6A immunostaining

was absent in 484 (22.8%), low (1+) in 371 (17.5%), moderate (2+) in 494 (23.2%), and strong (3+) in 776 (36.5%) of 2,125 analyzable urothelial bladder carcinomas. Examples of KDM6A stained tumor and normal urothelium samples are shown in Figure 1. As there were no significant differences between tumors with weak (1+), moderate (2+), and strong (3+) KDM6A positivity, all tumors with KDM6A positivity were grouped together as "KDM6A retained". A complete KDM6A expression loss occurred in 36% of 350 pTa G2 low-grade, 23% of 152 pTa G2 high-grade, and 18.5% of 92 pTa G3 tumors (p=0.0004). As compared to pTa G3 tumors, the frequency of KDM6A expression loss did not differ significantly in pT2 (17.2%), pT3 (21.9%), and pT4 (18.2%) cancers. Within pT2-4 carcinomas, KDM6A staining was unrelated to pT, pN, grade, L-status, V-status, and overall survival (Tab. II, Fig. 2a; p>0.1894). KDM6A loss was significantly related to low tumor cell proliferation as measured by Ki-67 IHC. The Ki-67 labeling index (Ki-67 LI) was 12.5±0.7 in 422 tumors with KDM6A loss compared to 17.3±0.4 in 1467 tumors with retained KDM6A expression (p<0.0001). This relationship was independent of the tumor stage (pTa: p=0.0627, pT1: p=0.013, pT2: p=0.0402, pT3: p=0.0004, pT4: p=0.0256, see Supplementary Fig. 1). KDM6A loss was more common in male (22.2%) than in female patients (15.4%; p=0.0067) and within male patients, and was more frequent in tumors with Y-chromosome loss (36.1%) than in cancers without Y-chromosome loss (16.3%; p<0.0001). This relationship was also retained within pTa tumors, where a KDM6A loss occurred in 31.8% of male but in only 20.8% of female patients (p=0.0239), and – in males – a KDM6A loss was seen in 57.8% of tumors with Y-chromosome loss but in only 25.4% of cancers without a Y-chromosome loss (p<0.0001, Tab. III). The absence of a prognostic impact of KDM6 expression loss was independent of whether the Y-chromosome was lost or not in muscle invasive cancers (Fig. 2b).

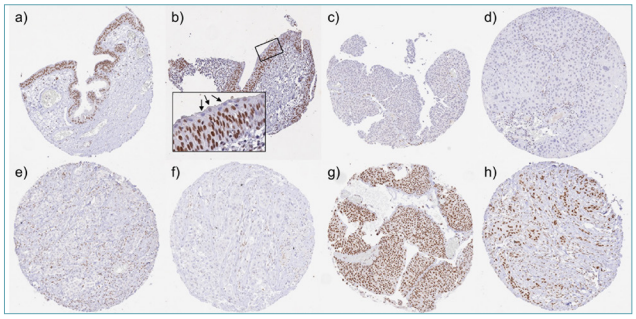

Figure 1. KDM6A staining. The panels show strong nuclear staining of (a-b) the normal urothelium of the urinary bladder, (b) often with less intense staining of the umbrella cells (indicated by arrows in the inset) as compared to the remaining epithelial cells, loss of KDM6A staining in (c) a non-invasive pTa low grade, in (d) a non-invasive pTa high grade carcinoma, and in (e-f) two muscle invasive urothelial carcinomas, and retained KDM6A staining in (g) a non-invasive pTa and (h) a muscle invasive carcinoma.

KDM6A MUTATION ANALYSIS

Data on both *KDM6A* mutation and KDM6A expres-

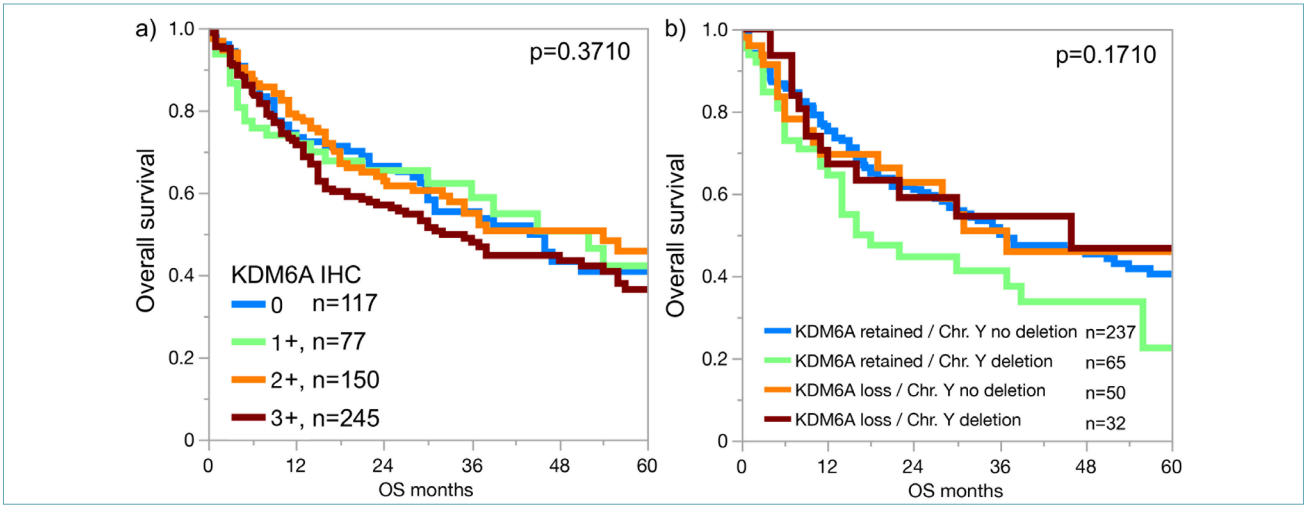

Figure 2. Lack of prognostic relevance of KDM6A expression in a) all muscle invasive (pT2-4) urinary bladder cancers and in b) subsets of tumors grouped after presence of absence of KDM6A expression and Y-chromosome loss.

Table II. KDM6A expression and bladder cancer phenotype.

		KMD6A IHC result		
	n	Loss (%)	Retained (%)	P
All cancers	2125	22.8	77.2	
pTa G1	5	60.0	40.0	
pTa G2 low	345	35.7	64.3	0.0004
pTa G2 high	152	23.0	77.0	
pTa G3	92	18.5	81.5	
pT2	378	17.2	82.8	0.1894
pT3	503	21.9	78.1	
pT4	247	18.2	81.8	
G2*	85	18.8	81.2	0.8466
G3*	1021	19.7	80.3	
pN0*	546	18.9	81.1	0.7870
pN+*	378	19.6	80.4	
R0*	463	21.2	78.8	0.4856
R1*	115	18.3	81.7	
L0*	209	17.7	82.3	0.4138
L1*	231	20.8	79.2	
V0*	355	18.3	81.7	0.1894
V1*	126	23.8	76.2	
Pn0*	55	20.0	80.0	0.4703
Pn1*	79	25.3	74.7	

Abbreviations: pT: pathological tumor stage, G: Grade, pN: pathological lymph node status, R: resection margin status, L: lymphatic invasion, V: venous invasion, PNI = perineural invasion; * pT2-4 cancers only

Table III. KDM6A expression, sex and Y chromosome loss.

				KDM6A IHC result		
Parameter	Status	Subset	n	Loss (%)	Retained (%)	p
Patient gender	Female	pT2-4	338	15.4	84.6	0.0067
	Male		920	22.2	77.8	
	Female	pTa	101	20.8	79.2	0.0239
	Male		478	31.8	68.2	
Y-chromosome	Y loss	male, pT2-4	238	36.1	63.9	< 0.0001
	No loss		607	16.3	83.7	
	Y loss	male, pTa	64	57.8	42.2	< 0.0001
	No loss		197	25.4	74.6	

sion were available on 78 tumors (Tab. IV). Truncating *KDM6A* mutations were seen in 48.7% and non-truncating *KDM6A* mutations in 5.1% of these selected cases. Truncating but not non-truncating *KDM6A* mutations were significantly associated with KDM6A expression loss ($p<0.0001$). This also held true for the subsets of 36 male ($p<0.0001$) and 42 female ($p=0.0011$) patients. All male patients with a truncating KDM6A mutation had complete KDM6 expression loss. There was, however, a small subset of tumors with a complete KDM6 expression loss that lacked truncating or even non-truncating *KDM6A* mutations both in male (n=5, 25%) and in female (n=4, 19%) patients. Images from these tumors with KDM6A expression loss in the absence of truncating *KDM6A* mutations are shown in Figure 3. In female patients, only 17 of 23 (73.9%) cancers with a truncating *KDM6A* mutation had complete KDM6A expression loss in the TMA spots. KDM6A IHC on the entire whole sections from the blocks used for sequencing of the remaining 6 cancers revealed a focal (heterogeneous) KDM6A staining loss in one of these tumors (Figs. 4a-b), a reduced but still significant KDM6A staining in at least half of the tumor tissue in 3 cancers (Fig. 4c), and normal (high-level) KDM6A expression in 2 tum-

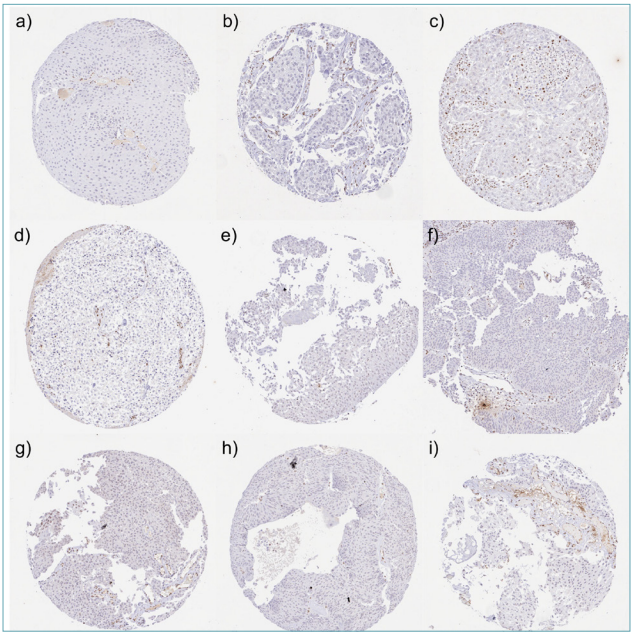

Figure 3. Examples of urothelial carcinomas with KDM6A expression loss but lack of truncating *KDM6A* mutations. a) pTa grade 2, female, *KDM6A* Glu1051Gln; b) pT2 grade 3, female, *KDM6A* wildtype; c-d) pT3 grade 3, female, *KDM6A* wildtype; e) pTa grade 2, male, *KDM6A* Gly1016Arg; f-h) pTa grade 2, male, *KDM6A* wildtype.

Table IV. *KDM6A* sequencing results.

Sex	Tumor stage	Grade	KDM6A IHC status	*KDM6A* mutation status	*KDM6A* mutation type	Biological significance
Female	pTa	G2	Loss	mut	Leu1340_Lys1345del	Truncating
Female	pTa	G3	Loss	mut	Ser876*	Truncating
Female	pTa	G2	Loss	mut	Lys685fs	Truncating
Female	pTa	G2	Loss	mut	Arg1127fs	Truncating
Female	pTa	G2	Loss	mut	Glu1051Gln	Unknown
Female	pT3	G3	Loss	mut	X1183_splice	Truncating
Female	pT2	G3	Loss	wt	wt	
Female	pT3	G3	Loss	mut	Ser689fs	Truncating
Female	pT3	G3	Loss	mut	Glu206*	Truncating
Female	pT2	G3	Loss	mut	X1392_splice	Truncating
Female	pT2	G3	Loss	mut	Ala391fs	Truncating
Female	pT2	G3	Loss	mut	Phe289fs	Truncating
Female	pT2	G3	Loss	mut	Pro1191fs	Truncating
Female	pT1	G2	Loss	mut	Glu1217_Phe1219del	Truncating
Female	pT3	G3	Loss	wt	wt	
Female	pT3	G3	Loss	mut	Gln329*	Truncating
Female	pT4	G3	Loss	mut	X129_splice	Truncating
Female	pT2	G3	Loss	mut	Pro1195fs	Truncating
Female	pT3	G3	Loss	mut	X1095_splice	Truncating
Female	pT3	G3	Loss	wt	wt	
Female	pT3	G3	Loss	mut	p.Trp127*	Truncating
Female	pTa	G3	Retained	mut	Thr584Met	Unknown
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G3	Retained	wt	wt	
Female	pTa	G2	Retained	mut	X189_splice	Truncating
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	mut	Asp1360fs	Truncating
Female	pTa	G2	Retained	mut	Gly359fs	Truncating
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	mut	X148_splice	Truncating
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G3	Retained	wt	wt	
Female	pTa	G2	Retained	mut	Thr842fs	Truncating
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	wt	wt	
Female	pTa	G2	Retained	mut	Gln805*	Truncating
Male	pTa	G2	Loss	mut	Tyr215fs	Truncating
Male	pTa	G2	Loss	mut	Gly1016Arg	Unknown
Male	pTa	G2	Loss	mut	Leu376fs	Truncating
Male	pTa	G2	Loss	mut	Gln1264*	Truncating
Male	pTa	G2	Loss	mut	Gln607*	Truncating
Male	pTa	G2	Loss	wt	wt	
Male	pTa	G2	Loss	mut	X944_splice	Truncating
Male	pTa	G2	Loss	mut	Gln369*	Truncating
Male	pTa	G2	Loss	wt	wt	
Male	pTa	G2	Loss	wt	wt	
Male	pTa	G2	Loss	mut	Leu617fs	Truncating
Male	pTa	G2	Loss	mut	X112_splice	Truncating
Male	pTa	G2	Loss	mut	Tyr228fs	Truncating
Male	pTa	G2	Loss	mut	Asn604fs	Truncating
Male	pTa	G2	Loss	mut	X189_splice	Truncating

Table IV. Follows from the previous page.

Sex	Tumor stage	Grade	KDM6A IHC status	*KDM6A* mutation status	*KDM6A* mutation type	Biological significance
Male	pTa	G2	Loss	mut	Ser1068*	Truncating
Male	pTa	G2	Loss	mut	Leu956*	Truncating
Male	pTa	G2	Loss	mut	Leu617fs	Truncating
Male	pTa	G2	Loss	wt	wt	
Male	pTa	G2	Loss	mut	Leu956*	Truncating
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	mut	Val1241del	Unknown
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	
Male	pTa	G2	Retained	wt	wt	

ors (Fig. 4d).

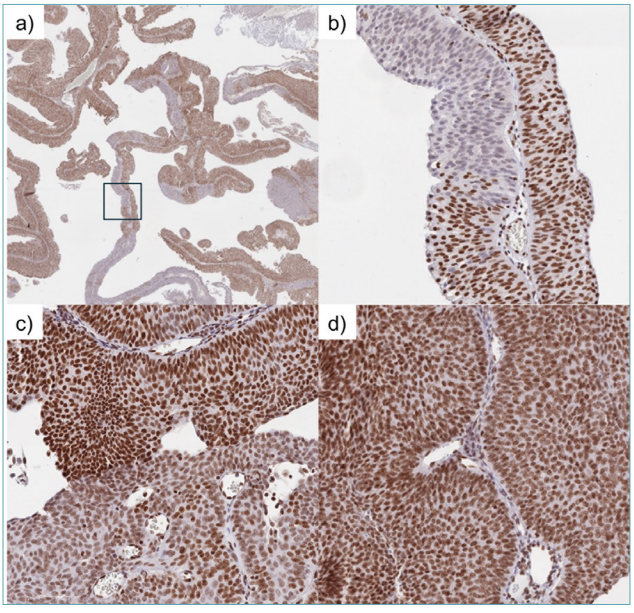

Figure 4. KDM6A immunostaining of conventional whole sections of a) a cancer with heterogeneous loss of KDM6A expression (overview) and b) magnification of the same tumor (see square in panel a). C) Tumor with focally reduced KDM6A staining. D) Tumor with strong KDM6A staining. All tumors harbored truncating *KDM6A* mutations.

Discussion

Our data show that KDM6A expression loss is a common event in urothelial neoplasms, especially in low grade cancers. While IHC data on KDM6A in urothelial cancer are so far lacking, our rates of KDM6A loss of 36% in pTa G2 low-grade, 23% in pTa G2 high-grade, 18.5% pTa G3, and 20% in pT2-4 cancers is in line with earlier data using next generation sequencing. These have also found a predilection of *KDM6A* alterations in low grade and non-invasive cancers and described *KDM6A* mutations in 40-52% of low-grade pTa, 30-38% of high-grade pTa, and in 23-25% of pT2-4 cancers [4,5]. The somewhat higher rate of *KDM6A* mutations compared to KDM6A expression loss is obviously because only deleterious (truncating) *KDM6A* mutations can be detected by IHC as "expression loss". In urothelial neoplasms, these made up for 69-80% of *KDM6A* mutations in males and for 62-82% in females in earlier studies [9-13].

Some functional studies had earlier suggested that KDM6A loss would result in cellular dedifferentiation [14], epithelial-mesenchymal transition [15], enhanced cell motility and invasiveness [16], high tumor burden and reduced survival [17], increased tumor aggressiveness [18], and loss of luminal tumor phenotype [14] of urothelial cancer cells. Considering the strong association with low grade and non-invasive cancer and with a low tumor cell proliferation index, as well as the complete lack of a prognostic role and of significant as-

sociations with parameters of aggressive disease in muscle-invasive cancers, our data do not support a role of KDM6A dysfunction for cancer progression in vivo. Our data suggest, however, a significant clinical utility of KDM6A IHC for the distinction of urothelial neoplasms from non-neoplastic urothelial cells. The identification of low-grade urothelial cancer - the subgroup with the highest rate of KDM6A expression loss - is challenging by morphology alone. This especially applies to flat lesions, small and crushed biopsies, and cytological specimens. KDM6A IHC can therefore complement more established markers to enable a safe diagnosis of low-grade urothelial neoplasms, at least in the subsets of patients with deficient KDM6A expression. Frequently used markers for this purpose, including CK20, p53, CD44, and (increasingly) MTAP [19], share the disadvantages of either a low prevalence in low-grade neoplasms or a lack of an unequivocal tumor association. Therefore, KDM6A IHC appears to be a much more appealing test, since KDM6A loss is directly associated with a cancer-causing genomic event.

That KDM6A, considered a major bladder cancer driver gene, is located on X chromosome is of particular interest. The potential disbalance of *KDM6A* redundancy between male and female patients has been proposed as a possible cause for the 3-fold higher prevalence of bladder cancer in males than in females [17]. In males, a catastrophic effect of a loss of X chromosome genes is often compensated by paralogs on the Y chromosome. Ubiquitous transcribed tetratricopeptide repeat containing Y-linked (*UTY*; *KDM6C*) is the *KDM6A* paralog on the Y chromosome. Similar as KDM6A, KDM6C can be incorporated into the COMPASS complex but has a slightly different function and lacks the demethylating properties of KDM6A [20]. That tumors with KDM6A loss had almost twice as many Y chromosome losses than tumors with retained KDM6A expression, both in our study and in a previous analysis by Ahn et al. [21], might suggest that the loss of both paralogs could result in an additional growth advantage of tumor cells. However, additional Y chromosome losses did not affect prognosis in tumors with a KDM6A expression loss in our study.

To assure similar expression levels as in males, one copy of X chromosome genes is normally repressed by imprinting in females. However, the imprinting mechanism is not 100% perfect and *KDM6A* is a gene that can escape X inactivation [22]. The significant but not perfect link between KDM6A expression loss and truncating *KDM6A* mutations in females is consistent with a significant but incomplete imprinting of *KDM6A*. That 17 of 23 cases with a truncating *KDM6A* mutation in females showed a complete KDM6A expression loss demonstrates that a loss of the active KDM6A allele cannot be compensated by the second allele in the majority of cases. Most likely, this is due to efficient imprinting of the second KDM6A allele in these cases. Since 5 of the 23 female patients with deleterious KDM6A mutation showed only a focally reduced or even “normal” KDM6A expression indicates, however, the second KDM6A allele is either not or only incompletely inactivated by imprinting and can compensate for the destructed allele in a minor fraction of cases. It is unlikely that the KDM6A mutation affected the inactive/imprinted gene copy as this would hardly result in a growth advantage of affected cells.

It was expected that all 15 tumors from male patients with truncating *KDM6A* mutations had a complete KDM6A expression loss which provides a strong indirect confirmation of our IHC assay. The fact that 25% of 20 male and 19% of 21 female cancers with complete KDM6A expression loss did not show a truncating *KDM6A* mutation demonstrates that at least one other mechanism can cause a KDM6A expression loss. Possible mechanisms with such an effect include mutation or methylation of the promoter region, histone modification and chromatin remodeling, transcriptional or post-transcriptional gene silencing by siRNAs, miRNAs, lncRNAs or other RNA binding proteins, genomic deletion, or combinations thereof [23-25]. A query of the cBioportal database for cancer genomics [26-28] including 11 studies [5,11,12,29-36] revealed that deep or shallow deletions (largely corresponding to homo- or heterozygous deletions) of the KDM6A locus can be found in 1% (deep deletion) and 8% (shallow deletion) of female bladder cancer patients lacking truncating KDM6A mutations. Irrespective of the mechanisms leading to KDM6A deficiency, tumors with a KDM6A expression loss may be candidates for treatment with EZH2 inhibitors [37]. KDM6A and EZH2 have opposing roles in regulating H3K27 methylation [38]. In KDM6A-mutant tumors, dysregulated EZH2 activity leads to excessive H3K27 methylation and transcription repression [39], causing tumor suppressor gene silencing, cancer initiation and progression [40]. There is evidence that inhibiting EZH2 in KDM6A-mutant cancers can restore histone methylation balance and reactivate tumor suppressor genes such as IGFBP3 [7] or IRF1 [41]. Functional analyses have shown that EZH2 inhibition results in decreased cell growth in *KDM6A*-null patient-derived cell line xenografts [7] and causes increased cell death in cell lines from muscle-invasive bladder cancers harboring *KDM6A* mutations [42]. A phase I clinical trial combining the EZH2 inhibitor tazemetostat with immune checkpoint therapy in 12 patients including individuals with advanced

urothelial carcinoma was published in 2019 and last updated in January 2023 (NCT03854474). The investigators reported partial response in 3 patients and stable disease in another 3 patients [43]. Although these tumors were not analyzed for *KDM6A* alterations, it is tempting to speculate that these responders included patients with *KDM6A* deficient cancers.

Conclusions

Our study identified KDM6A expression loss as a frequent molecular event in urothelial bladder cancer which preferentially occurs in low grade and non-invasive urothelial neoplasms. Truncating *KDM6A* mutations are the main but not the only cause for KDM6A expression loss. As KDM6A expression loss was not observed in non-neoplastic urothelium, we are now routinely applying KDM6A IHC to every newly diagnosed urothelial carcinoma and in case of flat urothelium with atypia of unknown significance. This procedure enables us to diagnose unequivocal dysplasia in a fraction of cases and to identify patients with *KDM6A* deficient tumors for which KDM6A IHC may be helpful for assessing subsequent biopsies or cytological specimens.

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Conflicts of interest statement

The mouse monoclonal antibody, clone HMV-314 was provided from ardoci GmbH (owned by a family member of GS)

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Authors' contributions

FV, NG, RS, GS, MK, SM: contributed to conception, design, data collection, data analysis and manuscript writing.
SB, AF, HP, SH, KF, SW, BR, AF, NB, BE, FR, SS, SE, ML, EB, AHM, HS, MF, MR, HZ, MS, KK, TE, SK, NA, JW, TK, TS, DH: participated in pathology data analysis, data interpretation, and collection of samples
FV, RS, MK, CHM: data analysis
FV, RS, GS, SM: study supervision
All authors agree to be accountable for the content of the work.

Ethical consideration

The use of archived remnants of diagnostic tissues for manufacturing of TMAs and their analysis for research purposes as well as patient data analysis has been approved by local laws (HmbKHG, §12) and by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

References

1 Comploj E, Trenti E, Palermo S, et al. Urinary cytology in bladder cancer: why is it still relevant? Urologia. 2015;82:203-205. https://doi.org/10.5301/uro.5000129

2 Wang G, Black PC, Goebell PJ, et al. Prognostic markers in pT3 bladder cancer: A study from the international bladder cancer tissue microarray project. Urol Oncol. 2021;39:e317-e328. https://doi.org/10.1016/j.urolonc.2021.01.021

3 Comperat E, Amin MB, Cathomas R, et al. Current best practice for bladder cancer: a narrative review of diagnostics and treatments. Lancet. 2022;400:1712-1721. https://doi.org/10.1016/S0140-6736(22)01188-6

4 Hurst CD, Alder O, Platt FM, et al. Genomic Subtypes of Non-invasive Bladder Cancer with Distinct Metabolic Profile and Female Gender Bias in KDM6A Mutation Frequency. Cancer Cell. 2017;32:701-715.e707. https://doi.org/10.1016/j.ccell.2017.08.005

5 Clinton TN, Chen Z, Wise H, et al. Genomic heterogeneity as a barrier to precision oncology in urothelial cancer. Cell Rep. 2022;41:111859. https://doi.org/10.1016/j.celrep.2022.111859

6 Hua C, Chen J, Li S, et al. KDM6 Demethylases and Their Roles in Human Cancers. Front Oncol. 2021;11:779918. https://doi.org/10.3389/fonc.2021.779918

7 Ler LD, Ghosh S, Chai X, et al. Loss of tumor suppressor KDM6A amplifies PRC2-regulated transcriptional repression in bladder cancer and can be targeted through inhibition of EZH2. Sci Transl Med. 2017;9:eaa8312. https://doi.org/10.1126/scitranslmed.aai8312

8 Minner S, Kilgue A, Stahl P, et al. Y chromosome loss is a frequent early event in urothelial bladder cancer. Pathology. 2010;42:356-359. https://doi.org/10.3109/00313021003767298

9 Damrauer JS, Beckabir W, Klomp J, et al. Collaborative study from the Bladder Cancer Advocacy Network for the genomic analysis of metastatic urothelial cancer. Nat Commun. 2022;13:6658. https://doi.org/10.1038/s41467-022-33980-9

10 Nguyen B, Fong C, Luthra A, et al. Genomic characterization of metastatic patterns from prospective clinical sequencing of 25,000 patients. Cell. 2022;185:563-575.e511. https://doi.org/10.1016/j.cell.2022.01.003

11 Pietzak EJ, Zabor EC, Bagrodia A, et al. Genomic Differences Between "Primary" and "Secondary" Muscle-invasive Bladder Cancer as a Basis for Disparate Outcomes to Cisplatin-based Neoadjuvant Chemotherapy. Eur Urol. 2019;75:231-239. https://doi.org/10.1016/j.eururo.2018.09.002

12 Kim P H, Cha E K, Sfakianos J P, et al. Genomic predictors of survival in patients with high-grade urothelial carcinoma of the bladder. Eur Urol. 2015;67:198-201. https://doi.org/10.1016/j.eururo.2014.06.050

13 Cancer Genome Atlas Research, N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature. 2014;507:315-322. https://doi.org/10.1038/nature12965

14 Barrows D, Feng L, Carroll T S, et al. Loss of UTX/KDM6A and the activation of FGFR3 converge to regulate differentiation gene-

expression programs in bladder cancer. Proc Natl Acad Sci U S A. 2020;117:25732-25741. https://doi.org/10.1073/pnas.2008017117

15 Xiao J F, Kua L F, Ding L W, et al. KDM6A Depletion in Breast Epithelial Cells Leads to Reduced Sensitivity to Anticancer Agents and Increased TGFbeta Activity. Mol Cancer Res. 2022;20:637-649. https://doi.org/10.1158/1541-7786.MCR-21-0402

16 Liu L, Cui J, Zhao Y, et al. KDM6A-ARHGDIB axis blocks metastasis of bladder cancer by inhibiting Rac1. Mol Cancer. 2021;20:77. https://doi.org/10.1186/s12943-021-01369-9

17 Kaneko S, Li X. X chromosome protects against bladder cancer in females via a KDM6A-dependent epigenetic mechanism. Sci Adv. 2018;4:eaar5598. https://doi.org/10.1126/sciadv.aar5598

18 Andricovich J, Perkail S, Kai Y, et al. Loss of KDM6A Activates Super-Enhancers to Induce Gender-Specific Squamous-like Pancreatic Cancer and Confers Sensitivity to BET Inhibitors. Cancer Cell. 2018;33:512-526.e518. https://doi.org/10.1016/j.ccell.2018.02.003

19 Gorbokon N, Wossner N, Lennartz M, et al. Prevalence of S-methyl-5'-thioadenosine Phosphorylase (MTAP) Deficiency in Human Cancer: A Tissue Microarray Study on 13,067 Tumors From 149 Different Tumor Types. Am J Surg Pathol. 2024. https://doi.org/10.1097/PAS.0000000000002297

20 Hong S, Cho Y W, Yu L R, et al. Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci U S A. 2007;104:18439-18444. https://doi.org/10.1073/pnas.0707292104

21 Ahn J, Kim K H, Park S, et al. Target sequencing and CRISPR/Cas editing reveal simultaneous loss of UTX and UTY in urothelial bladder cancer. Oncotarget. 2016;7:63252-63260. https://doi.org/10.18632/oncotarget.11207

22 Greenfield A, Carrel L, Pennisi D, et al. The UTX gene escapes X inactivation in mice and humans. Hum Mol Genet. 1998;7:737-742. https://doi.org/10.1093/hmg/7.4.737

23 Rinn J L & Chang H Y. Genome regulation by long noncoding RNAs. Annu Rev Biochem. 2012;81:145-166. https://doi.org/10.1146/annurev-biochem-051410-092902

24 Schwartz Y B & Pirrotta V. Polycomb complexes and epigenetic states. Curr Opin Cell Biol. 2008;20:266-273. https://doi.org/10.1016/j.ceb.2008.03.002

25 Hannon G J. RNA interference. Nature. 2002;418:244-251. https://doi.org/10.1038/418244a

26 Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012;2:401-404. https://doi.org/10.1158/2159-8290.CD-12-0095

27 Gao J, Aksoy B A, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013;6:pl1. https://doi.org/10.1126/scisignal.2004088

28 de Bruijn I, Kundra R, Mastrogiacomo B, et al. Analysis and Visualization of Longitudinal Genomic and Clinical Data from the AACR Project GENIE Biopharma Collaborative in cBioPortal. Cancer Research. 2023;83:3861-3867. https://doi.org/10.1158/0008-5472.CAN-23-0816

29 Van Allen EM, Mouw KW, Kim P, et al. Somatic ERCC2 mutations correlate with cisplatin sensitivity in muscle-invasive urothelial carcinoma. Cancer Discovery. 2014;4:1140-1153. https://doi.org/10.1158/2159-8290.CD-14-0623

30 Guo G, Sun X, Chen C, et al. Whole-genome and whole-exome sequencing of bladder cancer identifies frequent alterations in genes involved in sister chromatid cohesion and segregation. Nature Genetics. 2013;45:1459-1463. https://doi.org/10.1038/ng.2798

31 Robertson AG, Kim J, Al-Ahmadie H, et al. Comprehensive Molecular Characterization of Muscle-Invasive Bladder Cancer. Cell. 2017;171:540-556.e525. https://doi.org/10.1016/j.cell.2017.09.007

32 Iyer G, Al-Ahmadie H, Schultz N, et al. Prevalence and co-occurrence of actionable genomic alterations in high-grade bladder cancer. Journal of Clinical Oncology. 2013;31:3133-3140. https://doi.org/10.1200/JCO.2012.46.5740

33 Al-Ahmadie H, Iyer G, Lee BH, et al. Frequent somatic CDH1 loss-of-function mutations in plasmacytoid variant bladder cancer. Nature Genetics. 2016;48:356-358. https://doi.org/10.1038/ng.3503

34 Lee SH, Hu W, Matulay JT, et al. Tumor Evolution and Drug Response in Patient-Derived Organoid Models of Bladder Cancer. Cell. 2018;173:515-528.e517. https://doi.org/10.1016/j.cell.2018.03.017

35 Robinson BD, Vlachostergios PJ, Bhinder B, et al. Upper tract urothelial carcinoma has a luminal-papillary T-cell depleted contexture and activated FGFR3 signaling. Nature Communications. 2019;10:2977. https://doi.org/10.1038/s41467-019-10873-y

36 Guercio BJ, Sarfaty M, Teo MY, et al. Clinical and Genomic Landscape of FGFR3-Altered Urothelial Carcinoma and Treatment Outcomes with Erdafitinib: A Real-World Experience. Clinical Cancer Research. 2023;29:4586-4595. https://doi.org/10.1158/1078-0432.CCR-23-1283

37 Yamagishi M, Uchimaru K. Targeting EZH2 in cancer therapy. Current Opinion in Oncology. 2017;29:375-381. https://doi.org/10.1097/CCO.0000000000000390

38 Hemming S, Cakouros D, Isenmann S, et al. EZH2 and KDM6A act as an epigenetic switch to regulate mesenchymal stem cell lineage specification. Stem Cells. 2014;32:802-815. https://doi.org/10.1002/stem.1573

39 Tran N, Broun A, Ge K. Lysine Demethylase KDM6A in Differentiation, Development, and Cancer. Molecular and Cellular Biology. 2020;40. https://doi.org/10.1128/MCB.00341-20

40 Das P, Taube JH. Regulating Methylation at H3K27: A Trick or Treat for Cancer Cell Plasticity. Cancers (Basel). 2020;12. https://doi.org/10.3390/cancers12102792

41 Ezponda T, Dupère-Richer D, Will CM, et al. UTX/KDM6A Loss Enhances the Malignant Phenotype of Multiple Myeloma and Sensitizes Cells to EZH2 inhibition. Cell Reports. 2017;21:628-640. https://doi.org/10.1016/j.celrep.2017.09.078

42 Ramakrishnan S, Granger V, Rak M, et al. Inhibition of EZH2 induces NK cell-mediated differentiation and death in muscle-invasive bladder cancer. Cell Death & Differentiation. 2019;26:2100-2114. https://doi.org/10.1038/s41418-019-0278-9

43 Hussain MHA, Kocherginsky M, Singh P, et al. A pilot study of tazemetostat and pembrolizumab in advanced urothelial carcinoma (ETCTN 10183). Journal of Clinical Oncology. 2023;41:506-506. https://doi.org/10.1200/JCO.2023.41.6_suppl.506