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Loss of chromosome Y is unrelated to the composition of the tumor microenvironment and patient prognosis in muscle-invasive urothelial bladder cancers

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Abstract

Background Loss of chromosome Y (LOY) has recently been proposed to be associated with cancer aggressiveness, altered T-cell function, and poor prognosis in bladder carcinomas.

Methods Chromosome Y was analyzed using fluorescence *in-situ* hybridization on a tissue microarray containing 2,071 urothelial carcinomas of the urinary bladder from male patients, including 487 patients who had undergone cystectomy for muscle-invasive disease and for whom follow-up data were available. Data on tumor microenvironment were obtained from a previous study.

Results LOY was found in 26.0% of 1,704 analyzable cancers. In non-invasive cancers, LOY frequency was comparable in pTa G2 (22.8%) and pTa G3 (24.1%, $p = 0.8036$) carcinomas and slightly increased from pTa to pT2 - 4 carcinomas (23.1% for pTa and 27.2% for pT2 - 4) but these differences were not significant ($p = 0.0794$). In muscle-invasive cancers, LOY frequency slightly increased from pT2 (25.5%) to pT4 cancers (33.0%), but this association was not significant ($p = 0.1814$). Among pT2 - 4 cancers, LOY was associated with venous invasion ($p = 0.0010$) but unrelated to pT, pN, and L-status, as well as to overall, recurrence-free, and cancer-specific survival. Muscle-invasive urothelial carcinomas with and without LOY did not show significant differences in the number of CD8 positive lymphocytes, fraction of CD8 positive intraepithelial lymphocytes, number of macrophages and dendritic cells, and fraction of T helper and T regulatory cells.

Conclusion The lack of a clear association of LOY with histopathological parameters of cancer aggressiveness, patient prognosis, and parameters describing the tumor microenvironment strongly argues against the driving role of LOY in bladder cancer progression and cancer-associated immune reactions.

Keywords Loss of chromosome Y, Tissue microarray , Urothelial bladder cancer, Tumor microenvironment

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Introduction

Sex disparities in the incidence and mortality rates of cancer are well known. For most tumor entities, men have a higher incidence and mortality rate than women, including gastrointestinal, kidney, skin, brain, bladder, and head and neck carcinomas [1]. In addition, tumor entities with a higher incidence in women, such as breast and thyroid carcinomas, usually have a better survival rate than these tumor entities in men [1]. The reasons for these sex disparities have been extensively examined but are still not fully understood. For example, male tumors often have a higher mutation burden than female tumors [2–4]. The higher mutation burden may be explained by sex differences in the efficiency of the mismatch repair system in some tumor entities, including stomach or esophageal carcinomas [4]. However, there is also evidence that sex chromosomes influence tumor development and progression (summarized in [5]).

The Y chromosome includes various genes with a male-specific function, but also genes that are necessary for the regulation of gene transcription, mRNA translation, and protein stability, such as sex determining region Y (*SRY*), eukaryotic translation initiation factor 1 A Y-linked (EIF1 AY), and ubiquitin-specific peptidase 9 Y-linked (USP9Y) [6]. Loss of chromosome Y (LOY) is a common age-related event in males, which may be associated with a higher risk of developing some cancer types, such as leukemia, breast, and head and neck carcinomas [7–10]. In urothelial bladder cancer, Abdel-Hafiz et al. [11] recently proposed that LOY may represent a critical cancer-driving event associated with high cancer aggressiveness, poor survival, and altered T-cell function in muscle-invasive urothelial carcinoma. However, this is in contrast with several previous studies that failed to find associations between LOY and tumor phenotype or patient prognosis in cohorts of 16 to 477 noninvasive and muscle-invasive bladder carcinomas [12–19].

This study aimed to further assess the impact of LOY on tumor aggressiveness, patient prognosis, and composition of the tumor microenvironment in urothelial bladder cancer. Therefore, we analyzed chromosome Y copy number status by fluorescence *in-situ* hybridization (FISH) on a tissue microarray (TMA) containing 2,071 urothelial carcinomas of the urinary bladder from male patients, including 487 patients with muscle-invasive disease, and follow-up data after cystectomy. All of these tumors have been extensively analyzed for their immune cell composition.

Material and methods

Tissue Microarrays (TMA)

The TMAs were first employed in a study of the prognostic role of GATA3 expression measured by immunohistochemistry in urothelial bladder cancer [20]. All TMAs contained one sample each from urothelial bladder tumors of 2,071 male and 639 female (control) patients archived at the Institute of Pathology, Charité Berlin, Germany Institute of Pathology, University Hospital Hamburg, Germany, Department of Pathology, Helios Hospital Bad Saarow, Germany, or Department of Pathology, Academic Hospital Fuerth, Germany, and/or treated at the Department of Urology, Charité Berlin, Germany, Department of Urology, University Hospital Hamburg, Germany, Department of Urology, Albertinen Hospital, Hamburg, Germany, Department of Urology, Helios Hospital Bad Saarow, Germany, and Department of Urology and Urological Oncology, Pomeranian Medical University, Szczecin, Poland. At each center the patients were treated according to current guidelines. Shortly, transurethral bladder tumor resection with or without postoperative instillation therapy was used for pTa tumors. Radical cystectomy was applied for all pT2-pT4 cancers in our patient cohort. Table 1 shows all available histopathological data, including grade, tumor stage (pT), status of lymphatic (L) and venous (V) invasion, as well as lymph node status (pN). For 487 male patients with pT2 - 4 carcinomas who were treated with cystectomy clinical follow-up data were available as follow: overall survival—time between cystectomy and death (median: 16 months, range: 1–176 months, 487 patients), recurrence-free survival—time between cystectomy and recurrence (median: 11 months, range: 1–74 months, 185 patients), and cancer-specific death—time between cystectomy and cancer-specific death (median: 15 months, range: 1–77 months, 185 patients). All tumor tissues were fixed in formalin (4% buffered) and embedded in paraffin. The manufacturing process for TMAs has been described in detail, before [21, 22]. Shortly, one tissue spot (diameter: 0.6 mm) per tumor (patient) was removed. Data on 53 different tumor microenvironment parameters were obtained from a previous study [23].

Fluorescence *in-situ* hybridization (FISH)

TMA Sections. (5 µm cut thickness) were deparaffinized with xylol, rehydrated through a graded alcohol series, and exposed to heat-induced denaturation in a water bath at 99 °C for 10 min in P1 pretreatment solution (Agilent Technologies, Santa Clara, CA, USA; #K5799). For proteolytic treatment VP2000 protease buffer (Abbott, North Chicago, IL, USA; #2 J.0730) was used and slides were incubated at 37 °C in a water bath for 200 min. A commercial FISH probe kit containing centromere Y and X probes was used for copy number detection of chromosome Y (AneuVysion Multicolor DNA Probe Kit; Abbott, Chicago, IL, USA; #05 J38). Hybridization was made overnight at 37 °C in a humidified chamber.

Table 1 Patient cohort

	study cohort on TMA (n=2071)
follow up	487
months	
mean	26.4
median	16
tumor stage	
pTa	723 (42.8%)
pT2	322 (19.1%)
pT3	422 (25.0%)
pT4	222 (13.1%)
tumor grade	
G2	641 (31.9%)
G3	1366 (68.1%)
lymphnode metastasis	
pN0	535 (63.0%)
pN+	314 (37.0%)
resection margin	
R0	410 (80.9%)
R1	97 (19.1%)
lymphatic invasion	
L0	178 (48.2%)
L1	191 (51.7%)
venous invasion	
V0	293 (72.9%)
V1	109 (27.1%)

Percent in the column "study cohort on TMA" refers to the fraction of samples across each category. Numbers do not always add up to 2,071 in the different categories because of cases with missing data.

Post-hybridization washes were carried out according to the manufacturer's information (Agilent Technologies, Santa Clara, CA, USA; #K5799). Cell nuclei were dyed with 125 ng/ml 4',6-diamino- 2-phenylindole in antifade solution (Biozol; Eching, Germany; #VEC-H- 1200). Using an epifluorescence microscope, stained cell nuclei were manually interpreted and copy numbers of chromosomes Y and X were estimated for each TMA tissue core, as described before [13]. Absence of chromosome Y signal in all tumor cell nuclei but presence of one chromosome Y signal in normal cell nuclei and of at least one chromosome X signal in tumor and normal cell nuclei was considered loss of chromosome Y (LOY). Presence of two or more chromosome Y signals in at least 60% of all tumor nuclei and presence of one chromosome Y signal in normal cell nuclei were considered as gain of chromosome Y (GOY). The presence of one chromosome Y and one chromosome X signal in the tumor cell nuclei was considered as chromosome Y normal. For statistical analysis, tumor spots with GOY and normal chromosome Y status were summarized as non-deleted chromosome Y. The tumor spots of female patients were used for successful hybridization of the TMA slide. TMA spots without any detectable chromosome Y signal in all tumor and normal cells were excluded from the analysis because of the lack of an internal control for successful hybridization. Figure 1 shows examples of tumors with different Y chromosome copy number statuses.

Statistics

For statistical calculations JMP17® software (SAS®, Cary, NC, USA) was used. Chi-square test and contingency tables were used to search for associations between the copy number status of chromosome Y and tumor phenotype. The Kaplan–Meier method was used to calculate survival curves. Significant differences between the groups were estimated by the Log-Rank test.

Results

Technical issues

A total of 1,704 of 2,071 (82.3%) urothelial carcinoma tissue spots from male patients were informative in our chromosome Y FISH analysis. Reasons for non-informative cases (367 spots; 17.7%) included lack of tissue spots, insufficient hybridization with absence of chromosome Y and X signals in cancer and non-neoplastic nuclei, or absence of unequivocal cancer nuclei in the TMA spot.

Loss of chromosome Y (LOY) in urothelial carcinomas

LOY was detectable in 436 (25.6%) and GOY was detected in 471 (27.6%) of the 1,704 analyzable carcinomas. The fraction of LOY cancers was comparable in pTa G2 (22.8%) and pTa G3 (24.1%, $p = 0.8036$) carcinomas and slightly increased from pTa to pT2 - 4 carcinomas (23.1% for pTa and 27.2% for pT2 - 4) but these differences were not significant (0.0794; Table 2). In addition, there is no statistical difference between the frequency of LOY in pTa G3 (24.1%) and pT2 - 4 (27.2%; $p = 0.5391$) carcinomas. In muscle-invasive

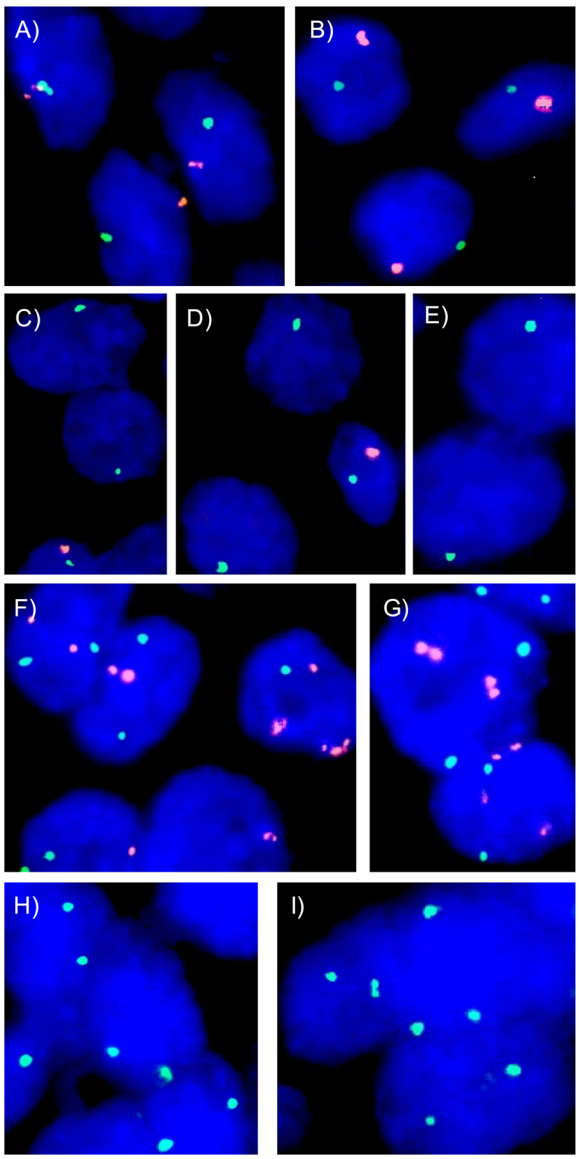

Fig. 1 Examples of chromosome Y copy number status. A/B) chromosome Y normal with one orange chromosome Y and one green chromosome X signal, C/D/E) loss of chromosome Y without any orange chromosome Y signal in tumor cell nuclei but one orange chromosome Y signal in adjacent normal cell nuclei and green chromosome X signals in both cell nuclei types F/G) gain of chromosome Y with ≥ two orange chromosome Y signal and two chromosome X signals, H/I) normal chromosome X status of female patients with two green chromosome X signals

Table 2 Loss of chromosome Y and tumor phenotype

Pathological Parameters	n	Copy number status of chromosome Y		p-value
		non-deleted	LOY	
All cancers	1704	74.4	25.6	
pTa G2 low	279	74.6	25.5	0.1542
pTa G2 high	124	83.1	16.9	
pTa G3	83	75.9	24.1	
pT2	282	74.5	25.5	0.1814*
pT3	378	72.8	27.3	
pT4	203	67.0	33.0	
G2	68	73.5	26.5	0.8851*
G3	1089	72.7	27.3	
pN0	477	74.2	25.8	0.3032*
pN+	284	70.7	29.2	
R0	374	71.1	28.9	0.9982*
R1	90	71.1	28.9	
L0	165	73.3	26.7	0.1569*
L1	175	66.3	33.7	
V0	276	73.5	26.5	0.0010*
V1	101	55.5	44.5	

Abbreviations: *LOY* loss of chromosome Y, *pT* pathological tumor stage, *G* Grade, *pN* pathological lymph node status, *R* resection margin status, *L* lymphatic invasion, *V* venous invasion, *only in pT2-4 urothelial carcinoma

cancers, the frequency of LOY increased also slightly from pT2 (25.5%) to pT4 cancers (33.0%), but this association was again not significant ($p = 0.1814$). In pT2-4 carcinomas, the frequency of LOY was associated only with V1 ($p = 0.0010$). No association was found between LOY and grade, pN, and L-status (Table 2), or with overall, recurrence-free, and cancer-specific survival (Fig. 2). Comparable results were obtained when tumors with GOY were separated, and tumors with LOY and normal chromosome Y copy number status ($n = 1$) were compared. In these analyses, the frequency of LOY was associated only with pT ($p = 0.0097$), L1 ($p = 0.0001$), and V1 ($p = 0.0200$; Table 3, Fig. 2D).

LOY and tumor microenvironment

Results on chromosome Y status were compared with parameters of the tumor microenvironment in 1,599 carcinomas from a previous study. In these analyses, LOY was not associated with the amount of CD3, CD4, CD8, and FOXP3 positivity on all, extra-tumoral, or intra-tumoral T-Cells in the tumor microenvironment (Fig. 3).

Discussion

In this study, we did not find a significant relationship between LOY and parameters of cancer aggressiveness, patient prognosis, or the composition of the tumor

Table 3 Loss and gain of chromosome Y and tumor phenotype

Pathological Parameters		Copy number status of chromosome Y			
	n	normal	LOY	GOY	p-value
All cancers					
pTa G2 low	279	60.6	25.5	13.9	0.0001*
pTa G2 high	124	60.5	16.9	22.6	0.1107**
pTa G3	83	39.8	24.1	36.1	
pT2	282	54.3	25.5	20.2	0.0027*
pT3	378	49.7	27.3	23.0	0.0097**
pT4	203	36.5	33.0	30.5	
G2	68	57.4	26.5	16.2	0.0068*
G3	1089	40.8	27.3	32.0	0.1998**
pN0	477	52.4	25.8	21.8	0.0144*
pN+	284	41.9	29.2	28.9	0.0537**
R0	374	48.4	28.9	22.7	0.8322*
R1	90	45.6	28.9	25.6	0.8273**
L0	165	57.0	26.7	16.4	0.0054*
L1	175	40.0	33.7	26.3	0.0200**
V0	276	53.6	26.5	19.9	0.0006*
V1	101	32.7	44.6	22.8	0.0001**

Abbreviations: *LOY* loss of chromosome Y, *GOY* gain of chromosome Y, *pT* pathological tumor stage, *G* Grade, *pN* pathological lymph node status, *R* resection margin status, *L* lymphatic invasion, *V* venous invasion, *normal, LOY, and GOY, **normal versus LOY

microenvironment of muscle-invasive urothelial bladder cancers.

LOY was found in 26% of 1,704 analyzable urothelial carcinomas in our TMA FISH analysis. This frequency is in the range of previous FISH studies showing LOY in 22% to 41% (mean: 26%) of 16 to 477 analyzed urothelial bladder carcinomas [12–19]. The fact that these data from different studies are rather similar is probably due to the high reproducibility of FISH data. FISH is the gold standard method for analyzing gene and chromosome copy numbers in tissues composed of mixed cell types, because a reliable morphology-based cell-by-cell analysis of the contained DNA copies can be executed. Therefore, most chromosomal Y studies have used FISH to determine the frequency of LOY in bladder carcinomas. A few studies evaluating DNA or RNA from disaggregated tissues to study Y chromosome copy numbers have found more variable and partly higher rates of Y chromosome loss ranging from 22 to 61% [11, 24].

LOY was only marginally related to some parameters of tumor aggressiveness in our analysis of 1,704 carcinomas. The slight increase in LOY from pTa G2 to pT4 cancers is consistent with a continuous loss of dispensable Y chromosomes during tumor evolution over time. The absence of significant associations between LOY and important clinicopathological parameters such as pT, pN, and survival within pT2 - 4 and the lack of statistical difference between the LOY frequency in pTa G3 and pT2 - 4 cancers argues against a significant cancer-promoting role of LOY in urothelial carcinoma. Our largely negative results are in line with data from previous FISH studies, which were also unable to find significant associations between LOY and overall survival in muscle-invasive and recurrence-free survival in non-muscle-invasive bladder cancer [13, 16, 18]. LOY determined by FISH was also unrelated to unfavorable tumor features or poor patient outcomes in cohorts of 1,045 renal cell carcinomas [25], 2,053 prostate adenocarcinomas [26], 30 esophageal

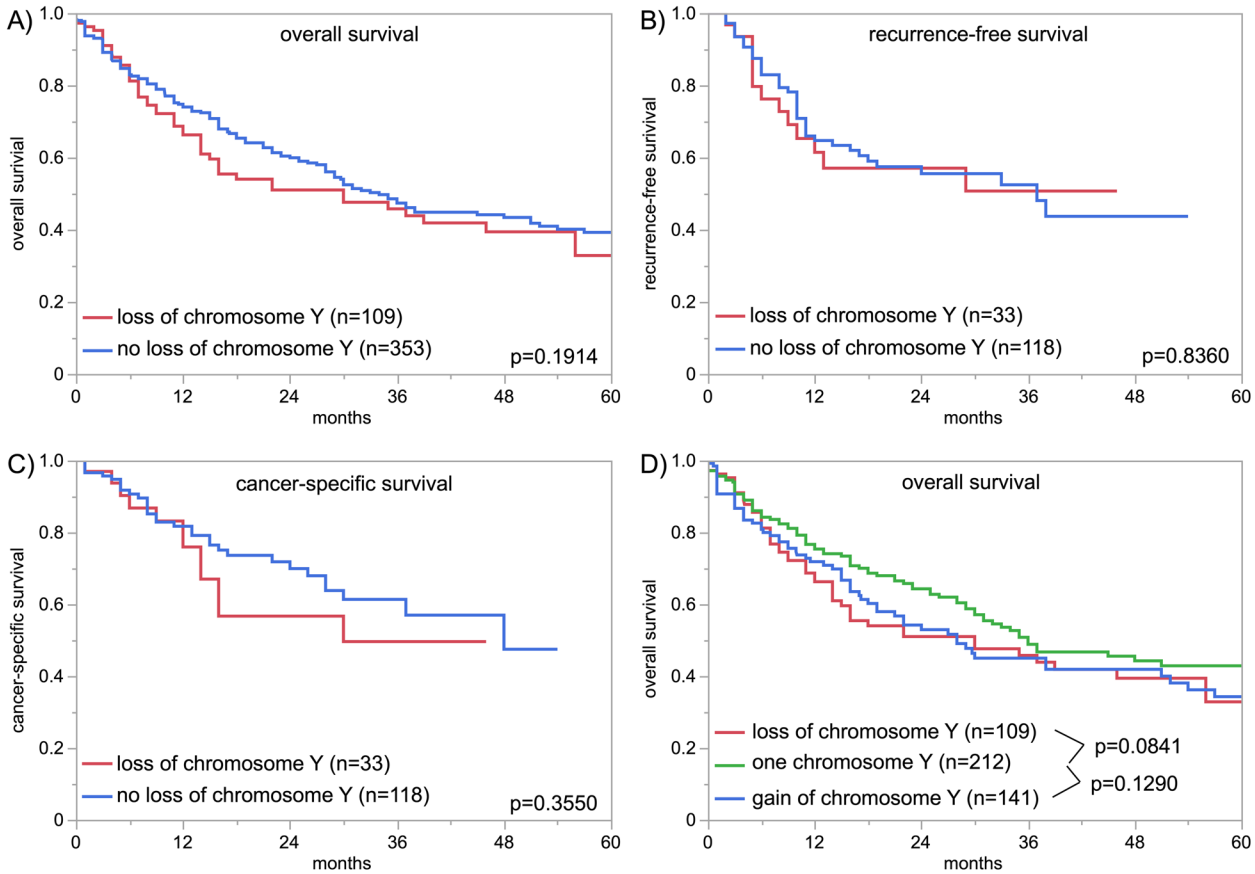

Fig. 2 Loss of chromosome Y and patient prognosis. **A**) loss of chromosome Y versus normal status of chromosome Y and overall survival, **B**) loss of chromosome Y versus normal status of chromosome Y and recurrence-free survival, **C**) loss of chromosome Y versus normal status of chromosome Y and cancer-specific survival, and **D**) loss of chromosome Y versus one chromosome Y versus gain of chromosome Y and overall survival

squamous cell carcinomas [27], and 35 hepatocellular carcinomas [28]. Overall, these data do not provide any evidence for a particular cancer-driving role for Y chromosome loss in cancer.

These data are in conflict with recently published data by Abdel-Hafiz et al., who proposed a marked prognostic role of LOY based on the analysis of the TCGA dataset by using a sophisticated algorithm depending on the expression of 18 Y chromosome genes, which resulted in a very high rate of 61% LOY cases. While this approach only resulted in a marginal prognostic difference ($p = 0.029$) in a cohort of 300 muscle-invasive urothelial carcinomas from the TCGA database, the authors further corroborated the notion of a cancer-driving role of LOY by a two-fold higher tumor growth rate for Y^- cells compared to Y^+ cells in immunocompetent mice. As a potential explanation for the differences between Y^- and Y^+ cells, the authors described a markedly suppressed immune response in seven mice with Y^- tumors compared to 10 Y^+ mice. In addition, Y^- cells in these mice responded better to anti-PD- 1 immunotherapy than Y^+ cells in mice in vitro [11].

Because of these data by Abdel-Hafiz et al., we were interested in the relationship between the tumor microenvironment and LOY in our patients. Using the same TMAs as in this study, we recently analyzed the prognostic role of the tumor microenvironment by multicolor immunohistochemistry using 21 different antibodies [23]. The delineation of 53 different immune cell phenotypes and their spatial relationship led to the identification of 32 immune parameters that were linked to prolonged overall survival ($p \leq 0.003$), 15 of which were independent of pT and pN stage. The validity of our approach was also supported by the strong prognostic role (among all analyzed parameters) of $CD8^+$ T cells, which were in direct contact with tumor cells. The extinction of tumor cells by cytotoxic T cells constitutes the intuitive terminal end route of the antitumor immune response. None of the tumor microenvironment and immune cell parameters were associated with LOY in 1,599 male patients

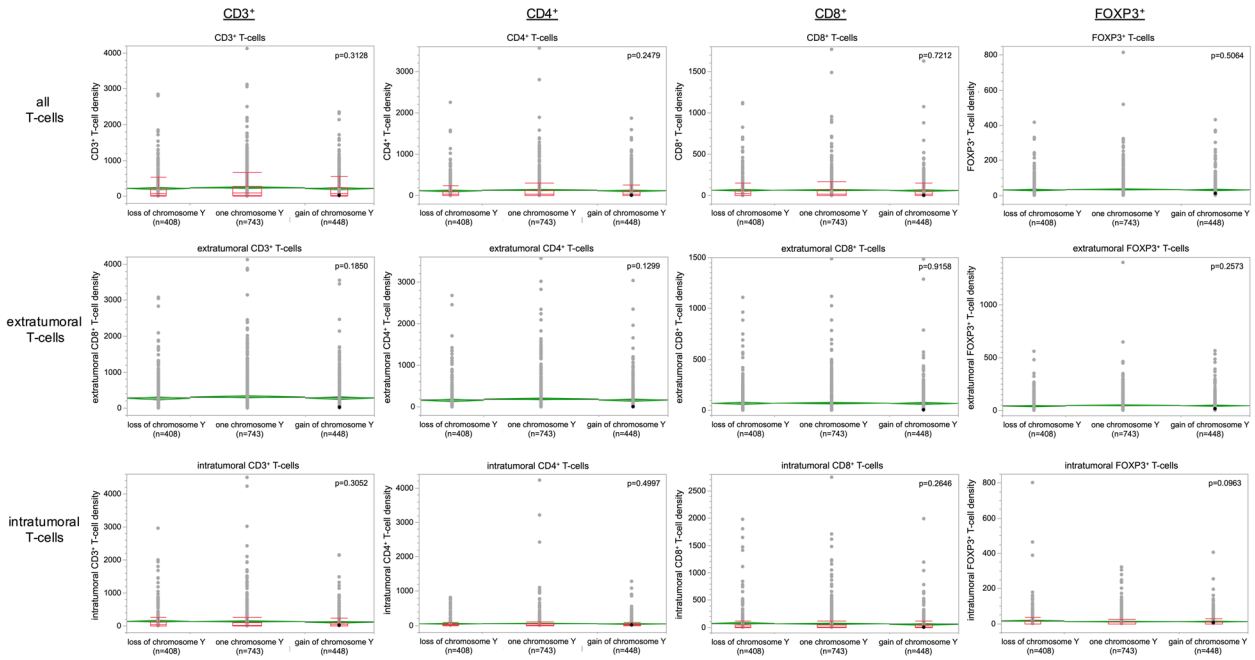

Fig. 3 Loss of chromosome Y and tumor microenvironement. The chromosome Y status was compared with the expression of CD3, CD4, CD8 and FOXP3 in all T-cells, extratumoral T-cells and intratumoral T-cells

with muscle-invasive urothelial carcinomas, which did not support the role of LOY in the immune escape of these tumors in vivo.

However, our study has also limitations. The number of examined tumors is still too small to investigate all difference between subgroups. Particular, a higher number of cases than 487 male patients with clinical follow-up data would be preferable. In addition, the lack of information on additional cancer treatment, including information about adjuvant or neoadjuvant chemotherapy as well as the retrospective nature of our analysis are further limitations of our study.

Conclusions

The lack of a clear association of LOY with histopathological parameters of cancer aggressiveness, patient prognosis, and parameters describing the tumor microenvironment strongly argues against the driving role of LOY in bladder cancer progression and cancer-associated immune reactions.

Abbreviations

EIF1 AY	Eukaryotic translation initiation factor 1 A Y-linked
FISH	Fluorescence *in-situ* hybridization
GOY	Gain of chromosome Y
L	Lymphatic invasion
LOY	Loss of chromosome Y
NGS	Next generation sequencing
pN	Lymph node status
pT	Tumor stage
SRY	Sex determining region Y
TCGA	The Cancer Genome Atlas
TMA	Tissue microarray
USP9Y	Ubiquitin specific peptidase 9 Y-linked
V	Venous invasion

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Authors' contributions

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

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All studies were conducted in compliance with the WMA Declaration of Helsinki – Ethical Principles for Medical Research involving Human Participants – 75 th WMA General Assembly, Helsinki, Finnland. The use of archived remnants of diagnostic tissues for manufacturing TMAs, their analysis for research purposes, and patient data analysis has been approved by local laws (HmbKHG, §12) and by the local ethics committee (Ethics commission Hamburg, WF- 049/09, Hamburg, Germany). Consent for participation from each patient is not necessary for this study based on the Ethics commission Hamburg (WF- 049/09).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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