



Reappraisal of *HER2* status in the spectrum of advanced urothelial carcinoma: a need of guidelines for treatment eligibility

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Abstract

Although human epidermal growth factor receptor 2 (*HER2*) may represent a therapeutic target, its evaluation in urothelial carcinoma of the bladder does not rely on a standardized scoring system by immunohistochemistry or fluorescent in situ hybridization (FISH), as reflected by various methodology in the literature and clinical trials. Our aim was to improve and standardize *HER2* amplification detection in bladder cancer. We assessed immunohistochemical criteria derived from 2013 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAPs) guidelines for breast cancer and investigated intratumoral heterogeneity in a retrospective multicentric cohort of 188 patients with locally advanced urothelial carcinoma of the bladder. Immunohistochemistry was performed on 178 primary tumors and 126 lymph node metastases, eligible cases (moderate/strong, complete/incomplete membrane staining) were assessed by FISH. *HER2* overexpression was more frequent with 2013 ASCO/CAP than 2007 ASCO/CAP guidelines ($p < 0.0001$). The rate of positive *HER2* FISH was similar between primary tumor and lymph node metastases (8%). Among positive FISH cases, 48% were associated with moderate/strong incomplete membrane staining that were not scored eligible for FISH by 2007 ASCO/CAP criteria. Among 3+ immunohistochemistry score cases, 67% were associated with *HER2*-positive FISH. Concordance between primary tumors and matched lymph node metastases was moderate for immunohistochemistry ($\kappa = 0.54$ (CI 95%, 0.41–0.67)) and FISH ($\kappa = 0.50$ (CI 95%, 0.20–0.79)). *HER2*-positive FISH was more frequent in micropapillary carcinomas (12%) and carcinoma with squamous differentiation (11%) than in pure conventional carcinoma (6%). Intratumoral heterogeneity for *HER2* immunohistochemistry was observed in 7% primary tumor and 6% lymph node metastases; 24% positive *HER2* FISH presented intratumoral heterogeneity. Our study suggests that *HER2* evaluation should include an immunohistochemistry screening step with eligibility for FISH including incomplete/complete and moderate/strong membrane staining. Spatial or temporal intratumoral heterogeneity prompts to perform evaluation on both tumor and lymph node, and for each histological variant observed.

Introduction

Urothelial carcinoma of the bladder is among the most frequent cancer in men in Europe and North America (fourth cancer in men and ninth in women), and muscle-invasive bladder cancer remains an aggressive disease. The search for

therapeutic targets and associated markers predictive of response is of critical importance. Among those potential targets, human epidermal growth factor receptor 2 (*HER2*) gene encodes for a transmembrane 185 kDa receptor tyrosine kinase protein, and its amplification is the primary mechanism for protein overexpression [1] being reported in 0–25% urothelial carcinoma of the bladder [2–17].

Although initial studies reported encouraging results, a randomized phase II trial failed to reach the required number of patients and was not able to confirm the effectiveness of trastuzumab (a humanized monoclonal antibody raised against *HER2*) in advanced or metastatic urothelial carcinoma [18]. This trial failure pointed out the need to screen a huge number of patients to identify those with *HER2* amplified

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tumors, and the need of an accurate *HER2* amplification rate for power calculation to design clinical trials. As fluorescent in situ hybridization (FISH) technology remains an expensive and time-consuming approach, an immunohistochemical step for screening is applied most frequently for breast [1] and gastric cancers [19]. Assessment of *HER2* status relies on a standardized scoring system using initial immunohistochemistry and FISH for confirmation.

In the absence of urothelial carcinoma standards, *HER2* immunohistochemistry in urothelial carcinoma of the bladder was scored according to breast cancer 2007 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAPs) guidelines in the majority of studies with complete membranous staining required for 2+ and 3+ immunohistochemistry scores [9–13, 15, 20, 21]. This approach was associated with significant false positive (9.5–52%) and negative rates (23–38.5%) suggesting an inadequate immunohistochemistry scoring system [13, 15, 17, 21, 22]. Interestingly, guidelines for breast cancer were revised in 2013 [23] to take into account incompletely immunoreactive membranes (“U” shaped), expanding the number of cases with a 2+ score submitted to FISH assay, and it is unknown whether this new scoring system might be effective in urothelial carcinoma. Overall, this lack of standardization of *HER2* testing in urothelial carcinoma of the bladder may account for the variable frequency reported for *HER2* overexpression and *HER2* amplification, and also impacts the inclusion in clinical trial and therapeutic efficacy evaluation [18, 24–26].

Using a cohort of locally advanced urothelial carcinoma of the bladder treated with radical cystectomy and adjuvant chemotherapy, we assessed immunohistochemical criteria derived from 2013 ASCO/CAP guidelines and investigated intratumoral heterogeneity to improve and standardize *HER2* amplification detection in bladder cancer.

Patients and methods

Study population

Our retrospective multicentric cohort including 226 consecutive patients with locally advanced urothelial carcinoma of the bladder treated with radical cystectomy and pelvic lymph node dissection followed by an adjuvant platinum-based chemotherapy regimen has been described previously [27].

Tissue samples and pathologic review

Paraffin-embedded tumor samples were available for 188 patients with 116 paired primary tumors and matched lymph node metastases. Pathological review according to

the WHO 2016 classification and TNM 2009 (7th edition) was performed by two pathologists (YA and AM) [28, 29].

Immunohistochemistry

Immunohistochemical staining was performed on 4- μ m thick formalin-fixed paraffin-embedded whole tissue sections using standardized automated methodology (Bond; Leica Menarini, Nanterre, France). The deparaffinized sections were stained for *HER2* (A0485, Dako, Glostrup, Denmark) after heat-induced antigen retrieval (dilution 1/500, pH6). The evaluation was carried out only on the invasive component of the tumor. External controls (absent, weak, moderate and strong *HER2* membrane staining on breast carcinoma samples) were included in the experiments.

In order to optimize the immunohistochemistry scoring procedure for bladder tissue, the following parameters were considered: percentage of immunoreactive tumor cells for each intensity of reactivity (classified as “absent”, “weak”, “moderate”, or “strong”) and degree of membranous reactivity (complete or incomplete). Finally, *HER2* immunohistochemistry scores were assessed according to 2007 ASCO/CAP [2] and 2013 ASCO/CAP guidelines [23] (Supplementary table 1). *HER2* intratumoral heterogeneity for *HER2* immunohistochemistry test was defined as two spatially distinct tumor areas, one with negative/weak staining, and the other (>10% tumor cells) with moderate/strong intensity.

Dual probe FISH analysis of *HER2*

All samples presenting at least 10% of cells with moderate or strong membranous staining (complete or incomplete) were evaluated by FISH. Fifty-nine primary tumors and 51 lymph node metastases were eligible for FISH analysis. Eight primary tumors and 18 lymph node metastases could not be assessed due to technical difficulties with the FISH analysis or to insufficient tumor material. FISH was performed on 3 μ m formalin-fixed paraffin-embedded whole tumor tissue sections using *HER2* FISH pharmDx kit (Dako, K533121).

Slides were analyzed with a Zeiss AxioImager Z1 (Zeiss, Göttingen, Germany) fluorescence microscope equipped with microscope-specific double filters (XF53; Omega Optical, Brattleboro, VT) suitable for the fluorescein isothiocyanate and Texas red-labeled signal probes. Images were captured with a $\times 40$ objective using a Hamamatsu digital camera attached to the fluorescence microscope and Visilog 6.9 software (FEI, Les Ullis, France).

Slides were analyzed with a $\times 100$ oil immersion objective. The numbers of chromosome 17 centromere (CEP17) and *HER2* signals were counted in 40

Table 1 Clinicopathological features of our 188 patients with advanced urothelial bladder cancer treated with adjuvant chemotherapy

Variable	<i>N</i> (%) or variable unit
Median age (years) at cystectomy (range)	62 (35–79)
Gender	
Female	31 (17)
Male	157 (83)
pT stage, <i>N</i> (%)	
T0	7 (4)
Tis	1 (1)
Ta	1 (1)
T1	4 (2)
T2	25 (13)
T3	94 (50)
T4	56 (29)
pN stage, <i>N</i> (%)	
Nx	18 (9)
N0	28 (15)
N1	45 (24)
N2	96 (51)
N3	1 (1)
Treatment	
Adjuvant chemotherapy regimen	
G	1 (1)
GC	131 (69)
GCb	45 (24)
MVAC	9 (5)
Other	2 (1)
Histological variant of primary tumors (<i>N</i> = 178)*	
Pure conventional urothelial carcinoma	68 (38)
Urothelial carcinoma with divergent differentiation	
Squamous differentiation	44 (25)
Glandular differentiation	4 (2)
Micropapillary carcinoma	50 (28)
Pure micropapillary carcinoma	20
Mixte micropapillary carcinoma	30
Plasmacytoid/single-cell carcinoma	13 (7)
Sarcomatoid carcinoma	23 (13)
Giant cell	1 (1)
Microcystic	1 (1)

A doxorubicin, G gemcitabine, C cisplatin, Cb carboplatin, M methotrexate, V vinblastine

*Sum of percentages above 100% due to tumor heterogeneity

non-overlapping nuclei. Only cells on which at least two CEP17 reference probe signals could be identified were included. For each case, the average *HER2* copy number, the average number of CEP17, and the ratio of *HER2* signals to CEP17 signals were calculated. According to 2013

ASCO/CAP *HER2* test guidelines, a *HER2*/CEP17 ratio ≥ 2.0 was reported as positive. When the *HER2*/CEP17 ratio was <2.0 , an average *HER2* copy number ≥ 6 was reported as positive, a copy number <4.0 was reported as negative, and a copy number in the range of 4.0–5.9 was reported as equivocal. Chromosome 17 polysomy was defined as ≥ 3 CEP17 signals per cell. For heterogeneous cases, histological variant, *HER2* immunohistochemistry scores, and *HER2* FISH status were reported for each component.

Statistical analysis

Frequencies and proportions were reported for categorical variables. The Chi2 Mc Nemar test measured the difference in immunohistochemistry results using 2007 and 2013 ASCO/CAP guidelines. A *p*-value < 0.05 was required for statistical significance. Cohen's kappa (with linear weighting) assessed the concordance of *HER2* status of the gene and protein between primary tumor and matched lymph node metastases.

Overall survival was assessed from the date of cystectomy to the date of death. The Kaplan–Meier method was used to visualize the survival distribution. The Cox proportional hazards model was used to estimate hazard ratio (HR) and test the statistical significance. The test was two-sided, and *p*-value < 0.05 was considered significant. The survival analysis was performed under R version 3.4.2.

Results

Patient characteristics

The clinicopathologic features of our cohort of 188 patients with advanced urothelial carcinoma of the bladder treated with radical cystectomy and adjuvant chemotherapy are listed in Table 1. Eighty percent of patients demonstrated pT3 or pT4 stage. Seventy-five percent of patients presented with lymph node metastases. Gemcitabine with cisplatin, gemcitabine with carboplatin, and methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) regimens were delivered in 131 (69%), 45 (24%), and 9 patients (5%), respectively. Primary tumors histological material was available for 178 patients. The most frequent histological variants were pure conventional urothelial carcinoma (38%), micropapillary carcinoma (28%), and urothelial carcinoma with squamous differentiation (25%).

HER2 protein expression and eligibility to HER2 FISH

Evaluation of *HER2* protein expression was performed by immunohistochemistry (Fig. 1, 2). We scored *HER2* protein

expression according to 2007 ASCO/CAP guidelines and observed a majority of score 1+ cases (103 primary tumors (58%) and 81 lymph node metastases (64%)) with only score 2 or 3 in 18 primary tumors (10%) and 15 lymph node metastases (12%). With the 2013 ASCO/CAP guidelines, the majority of cases presented a score of 2+ (93 primary tumors (52%) and 73 lymph node metastases (58%)) and we noticed an increase of the number of cases with the score 3+ (7 primary tumors (4%) and 4 lymph node metastases (3%)). Overall, the number of cases with HER2 protein overexpression (2+ or 3+ immunohistochemistry score) was statistically more important according to 2013 ASCO/CAP guidelines than the 2007 ASCO/CAP criteria ($p < 0.0001$, chi² Mc Nemar test).

In this study, we assessed *HER2* FISH status focusing in cases exhibiting a moderate or intense, complete or incomplete membrane staining in $\geq 10\%$ of tumors cells. Four cases presented intense complete circumferential intense staining in $< 10\%$ of tumor cells, but all were associated with a moderate staining justifying eligibility for FISH. With these criteria, the number of eligible cases for *HER2* FISH was intermediate between the 2013 and the 2007 ASCO/CAP guidelines (59 primary tumors (33%) and 51 lymph node metastases (40%)).

Correlation between HER2 gene status and HER2 protein expression

HER2 status could be determined by FISH in 51 primary tumors and 33 lymph node metastases (Fig. 1). *HER2* status was positive in 15 primary tumors (30%) and 10 lymph node metastases (30%), equivocal in 16 primary tumors (31%) and 11 lymph node metastases (33%), and negative in 20 primary tumors (39%) and 12 lymph node metastases (37%) (Supplementary Table 2 and Fig. 3). We compared *HER2* FISH status and HER2 immunohistochemistry score according to 2007 and 2013 ASCO/CAP guidelines (Table 2a and Supplementary table 3), 10 primary tumors and 4 lymph node metastases among them presenting heterogeneous HER2 immunohistochemistry scores. Among the 25 positive *HER2* FISH cases, eight primary tumors and four lymph node metastases had a score 1+, four primary tumors and five lymph node metastases score 2+ and three primary tumors and one lymph node metastases score 3+, in the 2007 ASCO/CAP system. The 12 cases with 1+ score exhibited incomplete membrane staining. With the more recent 2013 ASCO/CAP criteria, 12 primary tumors and 7 lymph node metastases showed 2+ staining and 3 primary tumors and 3 lymph node metastases had 3+ staining. Thirty primary tumors and 14 lymph node metastases were *HER2* FISH negative, none of them associated with 3+ staining in the 2007 and 2013 ASCO/CAP guidelines. Among 29 equivocal (16 primary tumors and 13

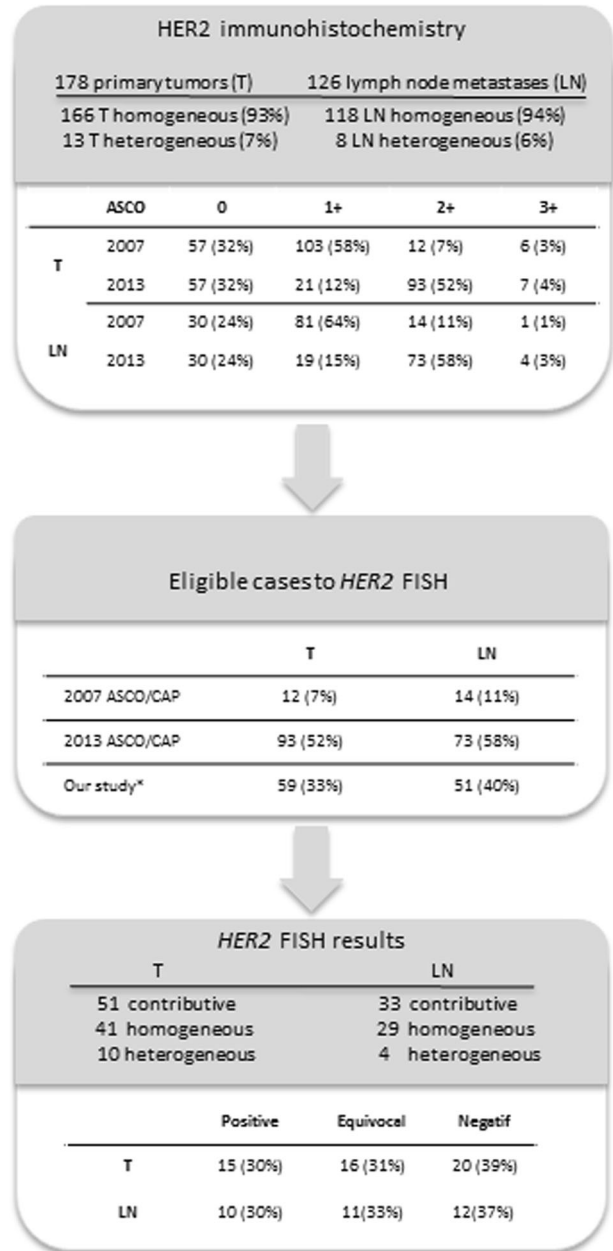


Fig. 1 Flowchart showing the three-step approach for *HER2* assessment in our cohort of locally advanced urothelial carcinoma of the bladder. * Eligible cases to FISH in our study: moderate or strong, complete or incomplete HER2 membrane staining in $\geq 10\%$ of tumor cells. ASCO/CAP American Society of Clinical Oncology/College of American Pathologists, FISH fluorescent in situ hybridization, HER2 human epidermal growth factor receptor 2, LN lymph node metastases, T primary tumor

lymph node metastases) and 44 negative (30 primary tumors and 14 lymph node metastases) *HER2* FISH cases, 24 (83%) and 23 (52%) cases showed polysomy, respectively. Overall, 54% primary tumors and 70% lymph node metastases presented polysomy with a mean CEP17 copy number 3.8 (± 0.9), and 3.7 (± 0.6), respectively.

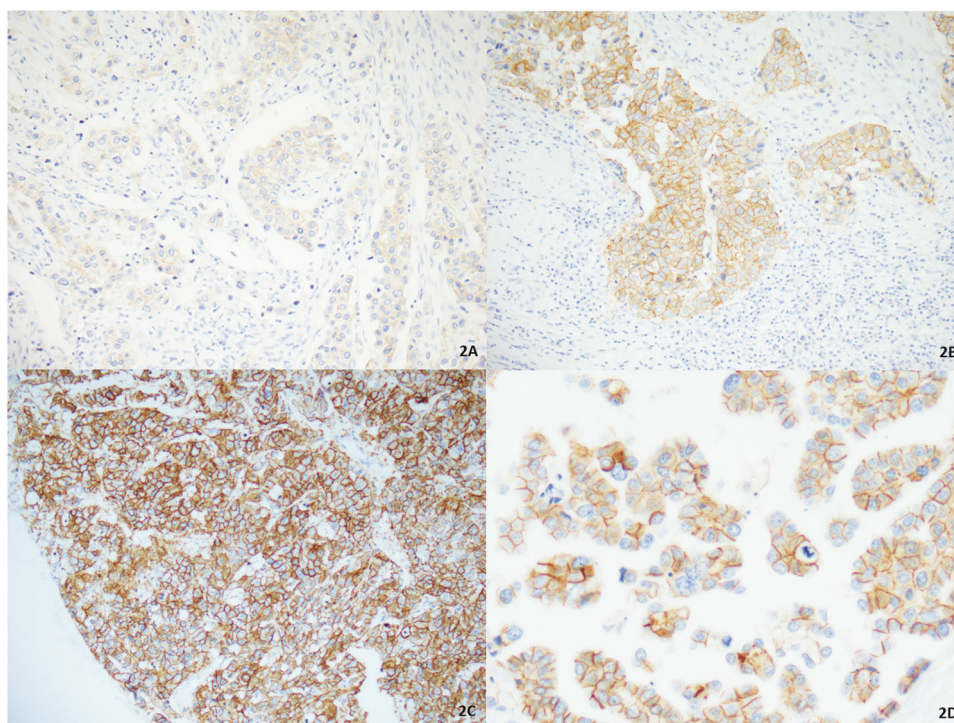


Fig. 2 HER2 expression in urothelial carcinoma by immunohistochemistry. **a** Weak complete membrane staining: score 1+ according to 2007 ASCO/CAP guidelines and score 2+ according to 2013 ASCO/CAP guidelines ($\times 200$). **b** Moderate complete membrane staining: score 2+ according to 2007 and 2013 ASCO/CAP guidelines ($\times 200$). **c** Strong complete membrane staining: score 3+ according to 2007 and 2013 ASCO/CAP guidelines ($\times 200$). **d** Moderate

incomplete (“U” shape) membrane staining in a micropapillary urothelial carcinoma. This case would be scored 1+ according to 2007 ASCO/CAP guidelines and 2+ with 2013 ASCO/CAP guidelines ($\times 400$). ASCO/CAP American Society of Clinical Oncology/College of American Pathologists, HER2 human epidermal growth factor receptor 2

Comparison of HER2 immunohistochemistry scores and HER2 FISH status between primary tumors and matched lymph node metastases

The HER2 immunohistochemistry score was evaluated for 116 paired primary tumors and matched lymph node metastases (Table 2b). As 2007 ASCO/CAP was less sensitive than the 2013 system, we focus thereafter on 2013 ASCO/CAP. Seventy-eight (67%) lymph node metastases exhibited the same score than corresponding primary tumor. Twenty-three (20%) lymph node metastases showed a higher HER2 immunohistochemistry score, whereas 15 cases (13%) had a lower HER2 immunohistochemistry score than the matched primary tumor ($\kappa = 0.54$ (confidence interval (CI) 95%, 0.41–0.67)). We evaluated *HER2* FISH status in 22 paired primary tumor and matched lymph node metastases (Table 2b). Fourteen (63%) cases exhibited concordant *HER2* FISH status between primary tumor and matched lymph node metastases ($\kappa = 0.50$ (CI 95%, 0.20–0.79)), whereas one primary tumor positive for *HER2* FISH assay was associated with a negative *HER2*

FISH lymph node metastases and conversely one negative FISH *HER2* primary tumor corresponded to positive *HER2* FISH lymph node metastases.

Association between HER2 gene status, HER2 protein expression, and histological variant

HER2 gene status and HER2 protein expression were studied according to the histological variant (Table 3). Cases with moderate/strong and incomplete/complete membrane staining represented 22%, 27%, and 23% of pure conventional urothelial carcinoma, urothelial carcinoma with squamous differentiation and plasmacytoid carcinoma, respectively. Pure micropapillary carcinoma displayed the highest proportion of eligible cases (15 primary tumors (75%)), whereas sarcomatoid carcinoma presented the lowest (3 primary tumors (13%)). Among 50 micropapillary urothelial carcinoma, 6 cases showed positive *HER2* FISH (12%). *HER2* FISH was positive in 6% pure conventional, 9% sarcomatoid, and 11% divergent differentiation with squamous feature urothelial carcinoma. None plasmacytoid carcinoma was positive.

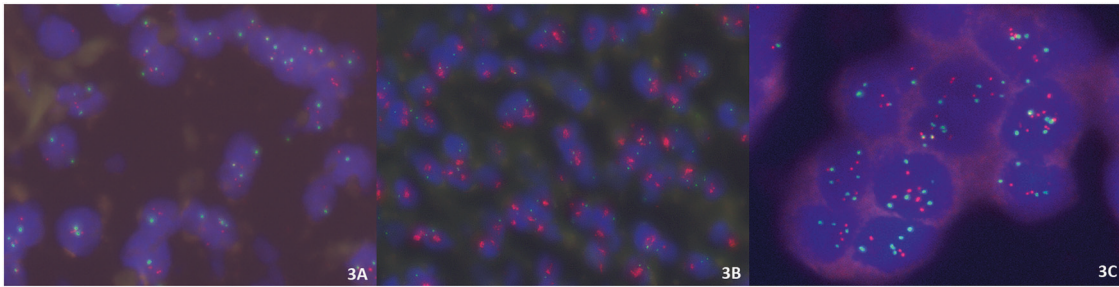


Fig. 3 *HER2* gene status in urothelial carcinoma. Red probe indicates *HER2* and green probe indicates chromosome 17 centromere. **a** Negative *HER2* FISH assay ($\times 400$). **b** Amplified/positive *HER2* FISH

assay with clusters of *HER2* gene signals ($\times 400$). **c** Polysomy for *HER2* ($\times 400$). FISH fluorescent in situ hybridization, *HER2* human epidermal growth factor receptor 2

HER2 heterogeneity

We identified *HER2* protein expression heterogeneity in 20 patients (13 primary tumors (7%) and 7 lymph node metastases (6%)) with 2 paired primary tumors and lymph node metastases both exhibiting *HER2* immunohistochemistry score heterogeneity (Table 4 and Fig. 4). Heterogeneous *HER2* protein expression corresponded to different histological variants in 13 cases among which 10 cases presented a combination of conventional and micropapillary urothelial carcinomas. *HER2* FISH status could not be assessed for six heterogeneous cases. Among the other 14 cases with *HER2* protein heterogeneity, we observed 10 cases with heterogeneous *HER2* FISH status: 5 negative/positive cases, 4 negative/equivocal cases, and 1 equivocal/positive case. Four cases with *HER2* protein heterogeneity demonstrated a homogeneous *HER2* FISH status (three cases with negative and one case with equivocal *HER2* FISH). *HER2* FISH assay was available in lymph node metastases for three primary tumors with a heterogeneous negative/positive *HER2* FISH assay. One lymph node metastases presented a positive *HER2* FISH, another showed an equivocal *HER2* FISH and the third one had a negative *HER2* FISH.

Prognostic impact of *HER2* status

Ten-year overall survival was not different between patients with *HER2* negative primary tumor and lymph node metastases versus patients with *HER2* positive/equivocal primary tumor or lymph node metastases (HR = 1.3; 95% CI (0.8–2.2), $p = 0.24$), although a trend for a worse outcome was associated with the positive/equivocal cases (Supplementary Figure 1).

Discussion

HER2 amplification detection is pivotal for patient eligibility to targeted therapy but various methodologies for

HER2 evaluation have been used in urothelial carcinoma of the bladder (immunohistochemistry screening or not, distinct criteria for *HER2* amplification) with a lack of standard criteria (see studies listed in supplementary table 4). In our study, we provided strong evidence supporting the use of immunohistochemistry criteria derived from updated breast cancer ASCO/CAP guidelines to optimize *HER2* amplification detection in urothelial carcinoma of the bladder. This methodology allowed us a reappraisal of *HER2* amplification in urothelial cancer in relationships with histological variants and intratumoral heterogeneity.

HER2 immunohistochemistry scoring is used to screen tumors potentially with *HER2* amplification, whereas FISH assay is considered as the gold standard. For breast and gastric cancers, positivity criteria have been optimized to provide a sensitive method. The latest 2013 ASCO/CAP guidelines for *HER2* evaluation in breast cancer considers (circumferential membrane staining that is incomplete and/or weak/moderate and within $>10\%$ of tumor cells) as 2+ immunohistochemistry score, whereas incomplete membrane staining was scored 1+ only according to 2007 ASCO/CAP guidelines. Similarly, incomplete moderate and strong membrane staining are, respectively, scored 2+ and 3+ in *HER2* scoring system for gastric cancer [19]. Our study was performed on locally advanced urothelial carcinoma of the bladder, which reflects better metastatic disease than earlier stages. We performed *HER2* FISH assay for cases presenting moderate/strong complete or incomplete membrane staining in $>10\%$ of cells, excluding only cases with weak complete/incomplete staining, and identified positive *HER2* FISH in $\sim 8\%$ cases. With the 2007 ASCO/CAP system scoring moderate incomplete staining as 1+, 12 positive *HER2* FISH cases would not have been assessed by FISH and diagnosed as not amplified. Unlike Lae et al., we observed also that complete moderate staining (2+ according to 2007 and 2013 ASCO/CAP guidelines) can be associated with positive *HER2* FISH [12]. Among nine cases with 3+ immunohistochemistry score according to 2013 ASCO/CAP guidelines, only six cases displayed positive *HER2* FISH,

Table 2 A) Comparison between HER2 immunohistochemistry score and HER2 FISH status in primary tumors and lymph node metastases, B) Comparison of HER2 status between primary tumors and matched lymph node metastases

A)						
FISH	HER2 IHC test according to 2007 ASCO/CAP guidelines					
	0 (N = 4)	1 + (N = 65)	2 + (N = 23)	3 + (N = 6)		
Positive (N = 25)	0	12 (18%)	9 (39%)	4 (67%)		
Equivocal(N = 29)	0	22 (34%)	5 (22%)	2 (33%)		
Polysomy(N = 24)	0	19	3	2		
Negative(N = 44)	4 (100%)	31 (48%)	9 (39%)	0		
Polysomy(N = 23)	2	15	6	0		
FISH	HER2 IHC test according to 2013 ASCO/CAP guidelines					
	0 (N = 4)	1 + (N = 4)	2 + (N = 81)	3 + (N = 9)		
Positive (N = 25)	0	0	19 (23%)	6 (67%)		
Equivocal(N = 29)	0	1 (25%)	25 (31%)	3 (33%)		
Polysomy(N = 24)	0	1	20	3		
Negative(N = 44)	4 (100%)	3 (75%)	37 (46%)	0		
Polysomy(N = 23)	2	1	20	0		
B)						
	IHC 2013 ASCO/ CAP	Lymph node metastases				Total
		0	1+	2+	3+	
Primary tumor	0	20	8	3	0	31
	1+	0	8	9	0	17
	2+	9	3	49	3	64
	3+	0	0	3	1	4
	Total	29	19	64	4	116
	FISH	Positive	Equivocal	Negative	Total	
	Positive	7	1	1 ^a	9	
	Equivocal	0	4	1	5	
	Negative	1 ^b	4	3	8	
	Total	8	9	5	22	

^a Heterogeneous primary tumor with homogeneous lymph node metastases

^b HER2 copy number per cell = 3.93 in primary tumor

ASCO/CAP: American Society of Clinical Oncology/College of American Pathologists, FISH: fluorescent *in situ* hybridization, HER2: Human Epidermal growth factor Receptor 2, IHC: immunohistochemistry.

whereas the three others showed equivocal *HER2* FISH, suggesting that *HER2* FISH assay remains required for strong complete membrane staining. “False positive 3+ immunohistochemistry score” urothelial carcinoma of the bladder have been reported also by Fleischmann et al. (9.5%) [13] and Schneider et al. (52%) [21].

The 2013 ASCO/CAP *HER2* guidelines for FISH included not only *HER2*/CEP17 ratio ≥ 2.0 but also *HER2* copy number as positive criteria. In our cohort assessed with the same 2013 guidelines, 20% of *HER2*-positive FISH were due to *HER2* polysomy (≥ 6 copies) independently of *HER2*/CEP17 ratio.

Our *HER2* amplification detection strategy derived from the 2013 ASCO/CAP guidelines for breast cancer allowed us to revisit its frequency according to bladder carcinoma histological variants. In our cohort of locally advanced urothelial carcinoma of the bladder, only 38% were pure conventional urothelial carcinoma underscoring the enrichment of variants. It was reported an overall 53% incidence rate of bladder cancer variants in a series of 302 patients treated by cystectomy [30]. Similarly, unusual morphological variants represented 40% cases in a cohort of locally advanced (74% pT2–T4 stage) high-grade urothelial carcinomas of the renal pelvis [31]. Previous studies

Table 3 HER2 immunohistochemistry score and HER2 FISH status according to histological urothelial carcinoma variants in primary tumors

Primary tumor	IHC ASCO/CAP 2013					FISH				
	Histological variant ^a					Contributive cases				
	0 N (%)	1 N (%)	2 N (%)	3 N (%)	Eligible cases ^b N (%)	Positive	Equivocal	Negative		
Pure conventional urothelial carcinoma (N = 68)	22 (32)	7 (10)	37 (55)	2 (3)	15 (22)	4	5	6		
UCB with squamous differentiation (N = 44)	19 (43)	3 (7)	19 (43)	3 (7)	12 (27)	5	3	4		
UCB with glandular differentiation (N = 3)	1 (33)	0	2 (67)	0	2	2	0	0		
Micropapillary carcinoma (N = 50)	7 (14)	10 (20)	30 (60)	3 (6)	30 (60)	6	7	12		
Pure micropapillary carcinoma (N = 20)	1 (5)	4 (20)	14 (70)	1 (5)	15 (75)	3	5	5		
Mixed micropapillary carcinoma (N = 30)	6 (20)	6 (20)	16 (53)	2 (7)	15 (50)	3	2	7		
Plasmacytoid/single-cell carcinoma (N = 13)	3 (23)	3 (23)	7 (54)	0	3 (23)	0	1	1		
Sarcomatoid carcinoma (N = 23)	15 (65)	2 (9)	6 (26)	0	3 (13)	2	0	1		
Giant cell (N = 1)	0	0	1	0	1	.	.	.		
Microcystic (N = 1)	1	0	0	0	0	.	.	.		

ASCO/CAP American Society of Clinical Oncology/College of American Pathologists, FISH fluorescent in situ hybridization, HER2 human epidermal growth factor receptor 2, IHC immunohistochemistry, UCB urothelial carcinoma of the bladder

^a Sum of percentages above 100% due to tumor heterogeneity

^b Eligible cases according to criteria applied in our study (moderate or strong, complete or incomplete membrane staining in ≥10% of cells)

underscored higher HER2 protein overexpression and HER2 amplification rate in micropapillary carcinomas [20, 21]. According to recent molecular classification of urothelial carcinoma of the bladder, micropapillary carcinoma is considered as luminal type [32], which has been associated also with HER2 protein overexpression [16, 33]. Our results showed 60% micropapillary carcinomas with HER2 protein overexpression, but only 12% showed positive HER2-positive FISH versus 6% in conventional urothelial carcinoma. These results confirmed another report of 15% and 9% of HER2 amplification for micropapillary carcinoma and conventional urothelial carcinoma matched on stage [21]. Hence HER2 protein overexpression in micropapillary carcinomas is linked only partly to HER2 amplification. A high frequency (40%) of HER2 mutation has been detected in micropapillary urothelial carcinoma [34] without protein overexpression, suggesting that HER2 mutations are not responsible for the discrepancy between protein overexpression and amplification in micropapillary carcinomas. In our cohort, urothelial carcinoma with squamous differentiation (viewed as basal/squamous-like urothelial carcinoma at molecular level) displayed also a significant rate of positive HER2 FISH (11%). Unlike Kim et al. who reported that 50% plasmacytoid carcinoma displayed positive HER2 FISH [35], none of the 13 plasmacytoid/single-cell carcinomas presented positive HER2 FISH. Of note, a comparative genomic hybridization on 25 plasmacytoid urothelial carcinoma revealed gains on chromosome 17q in 88% cases but only 14% showed HER2 amplification [36].

Intratumoral HER2 heterogeneity has been rarely studied in urothelial carcinoma of the bladder. Lae et al. reported HER2 protein heterogeneity in 35% of urothelial carcinoma of the bladder with a 3+ immunohistochemistry score (1.8% of cohort) with FISH showing exactly the same heterogeneous pattern with adjacent positive and negative areas [12]. HER2 intratumoral heterogeneity has been shown to be frequent in gastroesophageal [37] and breast cancers [38, 39] (5–69% and 5–40%, respectively). This wide range might reflect the absence of standardized definition for HER2 heterogeneity. The definition of HER2 heterogeneity in breast cancer has only recently been clarified in 2013 ASCO/CAP guidelines. In our study, HER2 intratumoral heterogeneity for the HER2 immunohistochemistry test was defined as two distinct tumor areas, one with negative/weak staining, and the other (>10% tumor area) with moderate/strong intensity and excluded mosaic pattern. Six to 7% of cases displayed HER2 protein heterogeneity, and HER2 protein heterogeneity was associated with HER2 FISH heterogeneity in 71% of cases. Interestingly, heterogeneous cases represent ~1/4 positive HER2 FISH (6/25), suggesting that intratumoral heterogeneity of HER2 expression may potentially contribute to

Table 4 Characteristics of primary tumors and matched lymph node metastases with HER2 heterogeneous expression

Case		Histological variant	Homogeneity for HER2 IHC score	HER2 membrane staining intensity	FISH
#1	T	Squamous differentiation	Heterogeneous	Absent	Negative
	T	Micropapillary		Moderate	Positive
	LN	Conventional	Heterogeneous	Weak	Equivocal
	LN	Micropapillary		Moderate	Positive
#2	T	Conventional	Heterogeneous	Weak	Negative
	T	Glandular differentiation		Moderate	Positive
	LN	Conventional	Homogeneous	Moderate	Equivocal
	LN	Micropapillary		Moderate	Equivocal
#3	T	Conventional	Homogeneous	Moderate	Negative
	T	Micropapillary		Moderate	Negative
	LN	Conventional	Heterogeneous	Weak	Negative
	LN	Micropapillary		Moderate	Equivocal
#4	T	Conventional	Heterogeneous	Weak	Negative
	T	Conventional		Moderate	Equivocal
	LN	Squamous differentiation	Heterogeneous	Weak	NC
	LN	Squamous differentiation		Moderate	NC
#5	T	Conventional	Heterogeneous	Weak	Negative
	T	Micropapillary		Moderate	Negative
	LN	Conventional	Homogeneous	Moderate	Negative
	LN	Micropapillary		Moderate	Negative
#6	T	Conventional	Homogeneous	Weak	NC
	T	Micropapillary		Weak	NC
	LN	Conventional	Heterogeneous	Weak	Negative
	LN	Micropapillary		Moderate	Positive
#7	T	Squamous differentiation	Heterogeneous	Weak	Negative
	T	Micropapillary		Strong	Positive
#8	T	Conventional	Heterogeneous	Weak	Negative
	T	Micropapillary		Strong	Equivocal
	LN	Micropapillary	Homogeneous	Moderate	NC
#9	T	Conventional	Heterogeneous	Weak	Negative
	T	Micropapillary		Moderate	Negative
#10	T	Conventional	Heterogeneous	Absent	Negative
	T	Conventional		Moderate	Negative
#11	T	Conventional	Heterogeneous	Absent	Negative
	T	Plasmacytoid		Moderate	Equivocal
	LN	Conventional	Homogeneous	Moderate	NC
#12	T	Sarcomatoid	Heterogeneous	Absent	Negative
	T	Squamous differentiation		Moderate	Positive
	LN	Sarcomatoid	Homogeneous	Moderate	Negative
LN	Squamous differentiation	Moderate		Negative	
#13	T	Conventional	Homogeneous	Moderate	Equivocal*
	LN	Conventional with extra capsular extension		Heterogeneous	Weak
	LN	Conventional carcinoma within sinuses		Moderate	Equivocal***

Only cases with at least one sample with contributive FISH are reported

#13, equivocal HER2/CEP17 ratio = *1.62, **1.77, ***1.96

FISH fluorescent in situ hybridization, HER2 human epidermal growth factor receptor 2, IHC immunohistochemistry, LN lymph node metastases, NC non-contributive, T primary tumor

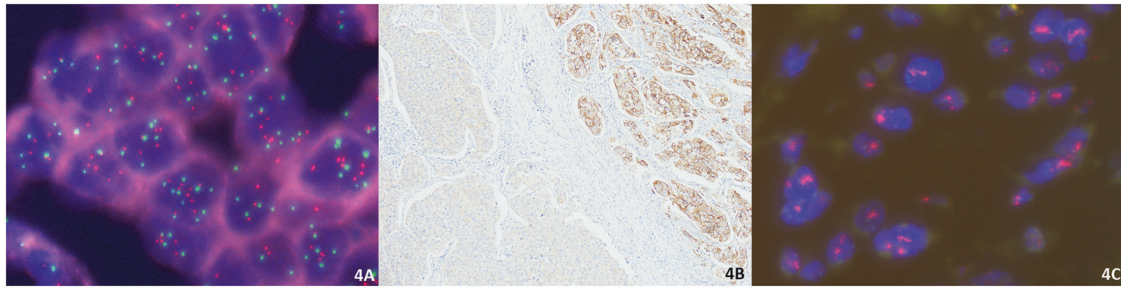


Fig. 4 HER2 heterogeneity in urothelial carcinoma. **a, c** *HER2* FISH (red probe indicates *HER2* and green probe indicates chromosome 17 centromere), **b** *HER2* immunohistochemistry. **b** Showing heterogeneity of staining in an urothelial carcinoma of the bladder. Moderate staining (2+) in micropapillary carcinoma (right side of image) adjacent to conventional carcinoma with faint staining (1+) (left side of

image) ($\times 100$). **a** Conventional carcinoma with faint staining was associated with negative *HER2* FISH assay (ratio = 1.06 and polysomy (mean centromere 17 copy/cell = 3.55)) ($\times 400$) whereas amplification of *HER2* was noted in micropapillary areas with *HER2* overexpression **c** ($\times 400$). FISH fluorescent in situ hybridization, *HER2* human epidermal growth factor receptor 2

inaccurate assessment of *HER2* status. *HER2* evaluation should be performed preferentially on cystectomy samples rather than transurethral resection of bladder tumor due to *HER2* intratumoral heterogeneity. Pathologists should be aware that *HER2* heterogeneity in urothelial carcinoma of the bladder is frequently associated with different co-existing histological variants, and give priority to samples with micropapillary carcinomas for *HER2* evaluation. Comparing primary tumor and matched metastatic lymph node, we observed *HER2* immunohistochemistry and *HER2* FISH score concordance in a majority of paired primary tumors and matched lymph node metastases. However, twenty-three (20%) lymph nodes showed a higher *HER2* immunohistochemistry score than the matched primary tumor suggesting that *HER2* should be evaluated systematically on primary tumor and lymph node metastases in urothelial carcinoma of the bladder. Three series investigated *HER2* status in lymph node-positive urothelial carcinoma of the bladder. Hansel et al. reported a higher concordance rate (88%) for *HER2* immunohistochemistry score between primary tumor and matched lymph node metastases [11]. More frequent *HER2* overexpression and *HER2* amplification in lymph node metastases than in primary tumor have also been reported [13, 40].

There are several limits to our study. We did not assess FISH on the whole cohort but on eligible cases based on immunohistochemistry results. As we did not investigate cases with weak and incomplete membranous staining, we cannot exclude that such staining is occasionally associated to positive *HER2* FISH in urothelial carcinoma of the bladder.

The results concerning *HER2* FISH status in lymph node metastases should be interpreted with cautiousness because 35% eligible lymph node metastases could not be assessed due to technical difficulties with the FISH analysis or to insufficient tumor material. Of note, only one non-contributive lymph node metastases corresponded to a positive *HER2* FISH primary tumor. We identified a higher

rate of equivocal cases (29%) than in breast cancer (1.9–9.5%) [41, 42] but it was beyond the scope of the study to perform an additional test to resolve these equivocal results.

Although phase II studies with anti-*HER2* therapies (Trastuzumab and Afatinib) [24, 25] demonstrated encouraging results, a randomized phase III study in stage IV urothelial carcinoma of the bladder patients with 2+ to 3+ immunohistochemistry score and/or Epidermal Growth Factor Receptor expression did not find significant improvements in outcome by the addition of maintenance lapatinib to standard of care [26]. Accurate assessment of *HER2* in urothelial carcinoma appears necessary to select properly eligible patients in clinical trials and to have reliable conclusions about anti-*HER2* therapy benefits in urothelial carcinoma of the bladder. Recently, Kiss et al. proposed an algorithm for molecularly stratifying urothelial carcinoma of the bladder patients for *HER2* targeted therapy, taking into account gene expression, amplification, polysomy, and somatic mutation [17]. However, we still do not know if these therapies should be proposed to *HER2* protein overexpressing or to *HER2* FISH-positive urothelial carcinoma of the bladder. As low level polysomy is a frequent event in urothelial carcinoma of the bladder, further studies will be necessary to assess the benefit of anti-*HER2* therapies in urothelial carcinoma of the bladder with *HER2* polysomy. It will be mandatory also to track *HER2* intratumoral heterogeneity to measure its impact on treated patients. Our study only focused on *HER2* protein expression and *HER2* gene status in locally advanced urothelial carcinoma of the bladder. We did not assess other potential drivers of advanced disease such non-amplifications *HER2* alterations, FGFR, or PDL1 expression. Interestingly, intratumoral CD8-positive cells, high expression of PD1, and absence of PDL1 expression may predict favorable response to anti-*HER2* therapies in *HER2*-positive breast cancers [43, 44]. Amplification of FGFR signaling may promote resistance to *HER2* inhibition [45]. Further studies

are required to identify predictive biomarkers to anti-HER2 therapies in urothelial carcinoma of the bladder.

Conclusions

Overall, our study suggests that HER2 evaluation should include an immunohistochemistry screening step with eligibility for FISH including moderate/strong and incomplete/complete membrane staining. To take into account the potential impact of spatial or temporal intratumoral heterogeneity, the evaluation should be performed on both tumor and lymph node, and for each histological variant observed. We hope that these recommendations will be helpful in assessing eligibility in future clinical trials.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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