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Evaluation of HER2 expression in urothelial carcinoma cells as a biomarker for circulating tumor cells

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Abstract

Background: Detection of circulating tumor cells (CTC) by techniques based on epithelial cell adhesion molecule (EpCAM) is suboptimal in urothelial carcinoma (UC). As HER2 is thought to be broadly expressed in UC, we explored its utility for CTC detection.

Methods: HER2 and EpCAM expression was analyzed in 18 UC cell lines (UCCs) by qRT-PCR, western blot and fluorescence-activated cell scanning (FACS) and compared to the strongly HER2-expressing breast cancer cell line SKBR3 and other controls. HER2 expression in UC patient tissues was measured by qRT PCR and correlated with data on survival and risk for metastasis. UCCs with high EpCAM and variable HER2 expression were used for spike-in experiments in the CellSearch system. Twenty-one blood samples from 13 metastatic UC patients were analyzed for HER2-positive CTCs with CellSearch.

Results: HER2 mRNA and protein were broadly expressed in UCC, with some heterogeneity, but at least 10-fold lower than in the HER-2+ SKBR3 cells. Variations were unrelated to cellular phenotype or clinicopathological characteristics. EpCAM expression was essentially restricted to UCCs with epitheloid phenotypes. Heterogeneity of EpCAM and HER2 expression was observed also in spike-in experiments. The 7 of 21 blood samples from 6 of 13 patients were enumerated as CTC positive via EpCAM, but only one sample stained weakly positive (1+) for HER2.

Conclusions: Detection rate of CTCs by EpCAM in UC is poor, even in metastatic patients. Because of its widespread expression, particularly in patients with high risk of metastasis, detection of HER2 could improve identification of UC CTCs, which is why combined detection using antibodies for EpCAM and HER2 may be beneficial.

KEYWORDS
circulating tumor cells, EpCAM, HER2, urothelial carcinoma

1 | INTRODUCTION

The current standard marker for the detection and for enrichment of circulating tumor cells (CTCs) in cancer patients is the epithelial cell adhesion molecule (EpCAM), a cell surface protein that has been shown to be expressed in a broad range of epithelial cancers (Andreopoulou et al., 2012; Gabriel, Calleja, Chalopin, Ory, & Heymann, 2016). Although, EpCAM-based detection of CTCs, for example, by using the approved CellSearch system, may be hampered substantially when EpCAM expression is diminished in epithelial cancer cells undergoing epithelial mesenchymal transition (EMT) (Gorges et al., 2012). As EMT has been observed to be both a key event in urothelial carcinoma (UC) pathogenesis (Garg & Singh, 2019) as well as a result of cytotoxic therapy (Skowron et al., 2015), this may explain why results reported on the EpCAM-based detection of CTCs in UC patients have not been convincing (Rink, Schwarzenbach, Riethdorf, & Soave, 2019).

HER2 (human epidermal growth factor receptor 2, gene name: *ERBB2*, erb-b2 receptor tyrosine kinase 2) could be a more suitable biomarker. Apart from UC, HER2 is overexpressed in a broad range of other human cancers, including gastric and ovarian cancer (Li & Li, 2016; Marchio, Balmativola, Castiglione, Annaratone, & Sapino, 2017; Ross & Gay, 2017; Urabe, Ushiku, Seto, & Fukayama, 2016; Weng et al., 2016). In UC, HER2 overexpression is associated with specific molecular subtypes of UC, namely with Cluster I and II in The Cancer Genome Atlas Network (TGCA) classification (Robertson et al., 2017) or with Uro and GU subtypes in the Lund system (Eriksson, Sjodahl, Chebil, Liedberg, & Hoglund, 2017). Further, as a negative prognosticator, HER2 overexpression has been linked with metastatic potential of UCs (Fleischmann, Rotzer, Seiler, Studer, & Thalmann, 2011; Nedjadi et al., 2016). Lastly, HER2 represents a well-druggable target. Antibodies and small molecule drugs against HER2 are prominently used to reduce tumor recurrence and cancer-related death in breast cancer (BC) (Burstein, Lieberman, Slamon, Winer, & Klein, 2005; Eisenhauer, 2001; Nielsen, Andersson, & Kamby, 2009). Although a recent phase III trial on the use of lapatinib—an inhibitor interrupting HER2/neu's tyrosine kinase activity—as a maintenance regime in patients with stable HER1/HER2-positive metastatic urothelial carcinoma (mUC) after first-line chemotherapy did not show any difference in survival rates (Powles et al., 2017). However, these results may be due rather to inconsistency in identification of HER2^{pos} patients (e.g., fluorescence in situ hybridization versus immunohistochemistry (Kiss et al., 2017) or immunohistochemistry versus expression on CTCs (Rink et al., 2012) than to lack of treatment efficacy.

Therefore, in the present study, we aimed to evaluate the suitability of HER2 as a biomarker in UC, especially focusing on the detection of CTCs. In detail, we checked the association of EpCAM and HER2 expression in a panel of commonly used UC cell lines (UCCs) by qRT-PCR, western blot analysis and flow cytometry. Results were compared to the strongly HER2-expressing BC cell line SKBR3. Further, HER2 expression was determined in a large cohort of UC patients with clinical follow-up data. To investigate the suitability of HER2 as a marker for CTC detection, we performed spike-in experiments with EpCAM- and HER2-positive UCCs using the CellSearch system to mimic clinical practice. Lastly, we analyzed blood samples of metastatic UC patients for HER2 expression of EpCAM positive CTCs.

2 | MATERIALS AND METHODS

2.1 | Patient populations

Thirteen patients with mUC at diagnosis, relapse after surgery or following previous chemotherapy were identified. From these patients, a total of 21 blood samples (7.5 mL each) were collected in CellSave tubes (Menarini Silicon Biosystems, Bologna, Italy) during follow-up at our institution, a tertiary referral center. Blood samples were collected before, at mid-term course and after completion of the chemotherapy cycle, if possible. Clinical data for these patients and a description of the samples are summarized in Table 1. The blood samples were analyzed for CTCs as described below.

A cohort of 144 bladder cancer tissues and 6 normal control tissues was analyzed for *ERBB2* gene expression by qRT-PCR. The majority of the patients was diagnosed with muscle-invasive bladder cancer; 44 pTa, 25 pT1, 18 pT2, 39 pT3, 18 pT4; 75 low grade and 69 high grade (Table 2). Median follow-up time for the complete cohort was 32 months and median follow-up of survivors was 68 months. About 88 of 144 patients have died during the follow-up period, 70 of 88 disease related. Statistical analysis was performed with the SPSS software (IBM, New York).

2.2 | Ethics approval and consent to participate

The study on CTCs was approved by the Ethics Committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf (study protocol numbers 4371 & 4192, approved August 18, 2016 and July 11, 2017). All patients signed an informed consent form.

Tissues samples for RNA extraction were collected according to the Declaration of Helsinki and with written patient informed consent as approved by the ethics committee of the medical faculty of the University Duisburg-Essen, Study Number 07-3537. The committee approved another amendment allowing comprehensive characterization of these tissues including the analysis of tissue RNA by qRT-PCR.

2.3 | Cell lines and cell culture

For this study, different human UCCs reflecting the heterogeneity of UC were used, namely RT112, BFTC905, RT4, SW1710, J82, 253J, 5637, 639v, HT1376, T24, UMUC3, VMCUB1, UMUC6, SD, 647v, Scaber, BC61 and BC44. Control cell lines comprised benign urothelial cell lines (HBLAK, spontaneously immortalized normal human bladder cell line and NHUC-TERT, TERT-immortalized normal human urothelial cells), the BC cell lines SKBR3 and MCF7, the prostate cancer (PC) cell line PC3 and the esophageal adenocarcinoma (OE) cell

TABLE 1 Patients characteristics, CellSearch results and HER2 IHC score

Patient ID	Indication for chemotherapy	Sample relative to chemotherapy	CellSearch CTCs count	HER2 in tumor sample	
				Primary tumor	Metastasis
URO1	M+	Pre	0	1+	
URO2	First recurrence	Pre first	0		
URO2	Second recurrence	Pre second	0		
URO2	Second recurrence	Mid second	0		
URO3	First recurrence	Pre	30	3+	3+ (PER)
URO4	First recurrence	Pre	78	3+	
URO4	First recurrence	Mid	0		
URO4	First recurrence	End	0		
URO5	N+	Pre	0	1+	1+ (PER, LN)
URO6	N+	Pre RCx and ACTx	0	1+	
URO8	M+	Pre	1		1+ (LN)
URO8	M+	Mid	1		
URO8	M+	End	0		
URO10	M+	Pre	1	0	
URO10	M+	Mid	0		
URO11	M+	Pre	1	1+	
URO12	M+	Pre	0	0	
URO13	M+	Pre	9 + cluster	0	1+ (HEP)
URO15	M+	Pre	0		
URO17	M+	Pre	0		

Abbreviations: ACTx, adjuvant chemotherapy; HEP, hepatic; LN, lymph node; PER, peritoneal; RCx, radical cystectomy.

lines OE19 and OE3. UCCs and OEs were cultured in DMEM GlutaMAX-I (Gibco, Thermo Scientific) supplemented with 10% FCS (Biochrom), PC3 was cultured in RPMI (Gibco). The SKBR3 cell line was cultured in McCoy's GlutaMAX-I (Gibco). NHUC-TERT cells were cultured in keratinocyte serum-free medium (Gibco) supplemented with 0.25 ng/mL EGF, 12.5 mg/mL bovine pituitary extract and 1:100 insulin-transferrin-selenium (Gibco), 0.35 mg/mL N-epinephrine, and 0.33 mg/mL hydrocortisone (Sigma-Aldrich, Munich, Germany). Cell lines were provided by Dr. M.A. Knowles (Leeds Institute of Cancer and Pathology, Leeds, United Kingdom), Dr. J. Fogh (Memorial Sloan-Kettering Cancer Center, New York, New York), Dr. B. Grossman (MD Anderson Cancer Center, Houston, Texas), Dr. N. Stoecklein (University Hospital Duesseldorf, Germany) and by the DSMZ (Braunschweig, Germany). Short-tandem repeat (STR) profiling was performed by standard DNA fingerprint analysis. The human bladder cell line HBLAK (Hoffmann et al., 2016), obtained from CELLnTEC, was cultured in CnT-Prime Epithelial Culture Medium (CELLnTEC). All cells were cultured at 37°C and 5% CO_2.

2.4 | RNA extraction, cDNA synthesis, and quantitative real-time PCR

High-quality RNA was extracted by the RNeasy Mini Kit as recommended by the manufacturer (Qiagen, Hilden, Germany). One microgram of RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was performed with QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Expression of EpCAM (EPCAM) and HER2 (ERBB2) mRNA was determined by qRT-PCR (primers, EPCAM forward: GCAGCTCAGGAAGAATG and reverse GCCAGCTTTGAGCAAATG; ERBB2 forward: CCTGGAACTC ACCTACCTGC and reverse CTGGGTGCCTCGCACAATC). The housekeeping gene TATA-box binding protein (TBP) was used as a reference gene (TBP forward ACAACAGCCTGCCACCTTA and reverse: GAATAG GCTGTGGGGTCAGT). Real-time qPCR was performed with the LightCycler 96 instrument (Roche, Mannheim, Germany) using initial denaturation at 95°C for 15 min and 45 cycles of amplification including denaturation at 95°C for 15 s, annealing at 61°C (ERBB2), 55°C (EPCAM) and 55°C (TBP) for 30 s and elongation at 72°C for 30 s.

2.5 | Cell separation and flow cytometry

Expression of cell surface markers EpCAM and HER2 was determined using a MACSQuant flow cytometer with the MACSQuant Analyzer 10 software (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were stained with antibodies against EpCAM (CD326-PE, #130-098-115, Miltenyi Biotec) or HER2 (HER2-FITC, CellSearch tumor phenotyping reagent HER2/neu, Menarini Silicon Biosystem), according to the manufacturer's instructions.

TABLE 2 Patient characteristics and *ERBB2* gene expression of the UC tissue specimen cohort

Variables	n	ERBB2*10,000 Median (range)	P
Age			
≤ 66	74	15.80 (0.83–488.59)	.108
> 66	70	13.88 (0.98–541.36)	
Gender			
Male	105	14.70 (0.83–541.36)	.952
Female	39	15.75 (0.98–277.43)	
Stage			
Ta	44	14.98 (0.83–68.69)	
T1	25	16.48 (3.19–52.59)	
T2	18	26.96 (3.83–488.59)	
T3	39	13.01 (1.64–541.36)	
T4	18	11.09 (0.98–277.43)	
Noninvasive	69	15.75 (0.83–68.69)	.763
Invasive	75	13.43 (0.98–541.36)	
Grade			
G1	26	15.71 (3.57–68.69)	
G2	49	16.07 (0.83–51.72)	
G3	69	12.34 (0.98–541.36)	
Low-grade (G 1–2)	75	16.07 (0.83–68.69)	.456
High-grade (G 3)	69	12.34 (0.98–541.36)	
LN/M status			
N0/Nx/M0/Mx	112	15.37 (0.83–541.36)	.773
N+/M+	32	13.44 (1.29–4.88.59)	
Smoking			
No	41	16.51 (3.72–277.43)	.168
Yes	36	12.06 (0.83–51.72)	
Unknown	67		
Control	6	8.31 (4.69–19.54)	.142
Tumor	144	14.79 (0.83–541.36)	

Abbreviations: UC, urothelial carcinoma.

2.6 | Protein extraction and western blot

To generate total protein lysates, cells were washed with phosphate-buffered saline (PBS) and directly lysed in buffer containing 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 50 mM Tris (pH 7.6), protease inhibitor cocktail (P-8340; Sigma-Aldrich) and phosphatase inhibitor cocktail (P-0044; Sigma-Aldrich) for 30 min on ice. Lysates were clarified by centrifugation.

For western blot analysis, equal amounts of protein were separated in SDS-PAGE gels and transferred to PVDF membranes (Millipore, Germany). Membranes were blocked with 5% nonfat milk in TBST (150 mM NaCl, 10 mM Tris, pH 7.4, and 0.1% Tween-20). HER2 primary antibody (1:1,000, Cell Signaling Technology, #2165)

was applied overnight at 4°C. Vinculin was detected as a loading control (Upstate/Merck, 05-386). After washing, the membrane was incubated with the suitable horseradish peroxidase-conjugated secondary antibody (Dako, Hamburg, Germany) for 1 hr and exposed using or WesternBright Quantum Kit (Biozym, Hessisch-Oldendorf, Germany) according to the manufacturer's recommendations. Signal intensities were recorded using a Bio-Rad ChemiDoc imaging system (Hercules, California).

2.7 | Spike-in experiment

Four blood samples (7.5 mL) were collected from a healthy donor in CellSave tubes (Menarini Silicon Biosystems), into which each 1,000 cells from SKBR3, RT112, 5637 and VMCUB1 cell lines were spiked. The CellSearch system (Menarini Silicon Biosystem) was used as previously described (Cristofanilli et al., 2004; Riethdorf et al., 2010). All blood samples were analyzed within 24 hr after spiking. Epithelial cells among the cells captured by anti-EpCAM antibodies were detected with antibodies directed against cytokeratins 8, 18, and 19. An anti-CD45 antibody was used to exclude leukocytes. Nuclei were counter-stained with DAPI. After enrichment and immunocytochemical staining, immunomagnetically labeled cells were immobilized in a strong magnetic field and scanned using the CellTracks Analyzer II (Menarini Silicon Biosystem). HER2 status of the CTC was assessed using the fluorescein-labeled anti-HER2 antibody (CellSearch tumor phenotyping reagent HER2/neu, Menarini Silicon Biosystem) and was categorized as negative (0), weakly positive (1+), equivocal (2+), or strongly positive (3+) as described (Cristofanilli et al., 2004). Image galleries were manually evaluated for CTC according to criteria reported earlier (Cristofanilli et al., 2004; Riethdorf et al., 2010) and by the ACCEPT (Automated CTC Classification Enumeration and PhenoTyping) Software as previously described (Zeune et al., 2017). Briefly, images generated during CellSearch analysis were re-analyzed using ACCEPT software. Objects of interest were defined based on shape contours and signal intensity and were automatically selected by an underlying algorithm. The marker characterization tool of ACCEPT allows to reevaluate the marker expression of prescored cells. A detailed description of the rescaling procedure of staining signals to their true intensity values before brightness adjustment by the CellSearch system is given in the study by Cristofanilli et al. (2004).

3 | RESULTS

3.1 | HER2 and EpCAM expression in UC cell lines

To characterize EpCAM and HER2 expression in UCC line models, 18 UCC lines (RT112, BFTC905, RT4, SW1710, J82, 253J, 5637, 639v, HT1376, T24, UMUC3, VMCUB1, UMUC6, SD, 647v, Scaber, BC61, BC44), as well as the immortalized non-neoplastic urothelial cell lines HBLAK and NHUC-TERT cells were screened for *EPCAM* and *ERBB2* mRNA levels by qRT-PCR. Breast cancer cell lines SKBR3 (HER2

amplified) and MCF7 (HER2 negative), the PC cell line PC3 and the esophageal adenocarcinoma cell lines OE19 and OE33 were used as controls. Information on copy number changes in cancer cell lines was obtained from the CCLE database (Broad Institute, Table 3).

EPCAM expression ranged from 0 to 25.12 relative units. Its mRNA was undetectable in J82, 253 J, 639v, T24 and UMUC3 cell lines, all of which have a mesenchymal phenotype (Figure 1a, Table 4). Instead, VMCUB1, RT4, BC61, 647v and SD, all of which have an epithelial phenotype, had the highest *EPCAM* expression levels across UC cell lines. In both benign control cell lines, HBLAK and NHUC-TERT, *EPCAM* expression was very low. The BC cell lines displayed intermediate expression levels, while *EPCAM* expression was the highest in the prostate and esophageal adenocarcinoma cell lines.

Unlike *EPCAM*, *ERBB2* mRNA was well detectable in all cell lines, albeit with different extensities (Figure 1b). Specifically, RT112, RT4, BC61, 647 and SD cell lines had the highest expression levels among

TABLE 3 Copy number for *ERBB2* gene in cancer cell lines from urinary tract, breast, prostate, and oesophagus according to CCLE database (https://portals.broadinstitute.org/ccle)

Cell line	ERBB2 copy number
Control cell lines	
SKBR3_BREAST	2.7814
MCF7_BREAST	−0.9661
PC3_PROSTATE	−0.5279
OE33_OESOPHAGUS	2.7228
OE19_OESOPHAGUS	5.2985
Bladder cancer cell lines investigated in this study	
639V_URINARY_TRACT	−0.0532
VMCUB1_URINARY_TRACT	0.119
RT112_URINARY_TRACT	−0.0438
SCABER_URINARY_TRACT	−0.1388
SW1710_URINARY_TRACT	0.3694
UMUC3_URINARY_TRACT	−0.1494
BFTC905_URINARY_TRACT	0.1682
HT1376_URINARY_TRACT	−0.2863
RT4_URINARY_TRACT	0.0819
647V_URINARY_TRACT	0.0841
Cell lines not investigated in this study	
RT11284_URINARY_TRACT	−0.0266
HT1197_URINARY_TRACT	−0.1076
UMUC1_URINARY_TRACT	0.1004
TCCSUP_URINARY_TRACT	−0.0626
JMSU1_URINARY_TRACT	−0.1004
SW780_URINARY_TRACT	0.1142
HS172T_URINARY_TRACT	−0.0162
CAL29_URINARY_TRACT	0.0464
KU1919_URINARY_TRACT	−0.1352
BC3C_URINARY_TRACT	−0.0894

UC cell lines. According to CCLE database results, no UC cell line included in the database harbored significant copy number changes for *ERBB2*. Concurrently, PC3 and MCF7 cells have no *ERBB2* amplification and thus expressed *ERBB2* at similar levels as UCC cells (Table 3). In contrast, *ERBB2* expression was by far highest in the BC control cell line SKBR3 and the two esophageal adenocarcinoma cell lines. These three cell lines carry *ERBB2* amplifications and our result of highest expression in the OE19 cell line corresponded well with the highest copy number gain in this cell line.

HER2 protein expression was analyzed in UCC representing the clinical spectrum of UC, for example, RT112 and SW1710 are derived from papillary UC; 253J was derived from a nodal metastasis; VMCUB1, 5637 and T24 from bladder UC; 639v from a ureteral UC; NHUC-TERT and HBLAK are benign cell lines. The BC cell line SKBR3 with its known *ERBB2* gene amplification was included as a positive control and reference for HER2 staining intensity (Nielsen et al., 2009), likewise OE19, OE33 and PC3. Of note, SKBR3 cells also expressed EpCAM (Schneck et al., 2015). Western blot analysis revealed again that even though HER2 is not amplified in UCC lines, the protein can be detected albeit to variable extent, but at a much lower level compared to HER2 amplified SKBR3 cells (Figure 2). Comparing UCC lines und benign controls, no obvious difference regarding HER2 expression was observed.

To assess the heterogeneity of expression within each cell line, we further analyzed HER2 expression by fluorescence-activated cell scanning (FACS) analysis (Figure 3a). For comparison, we investigated the expression of EpCAM protein and the number of EpCAM-positive cells by FACS (Figure 3b).

As expected, >90% of cells from the phenotypically epithelial UCC were EpCAM-positive. Conversely, only a small fraction (range 0.13–4.46%) of UCC with a mesenchymal phenotype were found to be EpCAM-positive (Table 4). SW1710, with its intermediate E/M phenotype, was the only exception with 95% EpCAM-positive cells. Thus, EpCAM expression in UCC is highly dependent on cellular phenotype.

For HER2, FACS revealed again a heterogeneous expression. Expression of HER2 tended to be higher in UCC with epithelial morphology, but was also detectable in UCC with mesenchymal phenotype. In line with mRNA and western blot data, every UCC line contained at least some HER2-positive cells. However, only two cell lines, namely BFTC905 and RT112 with epithelial-like morphology, consisted exclusively of HER2-positive cells, like SKBR3 cells (Table 4). Even these cell lines exhibited significantly less staining intensity (BFTC905 RFMI 8.3 [median fluorescence intensity], RT112 RFMI 15.7) than SKBR3 cells (RFMI 35.5).

3.2 | HER2 expression in urothelial cancer tissues

ERBB2 gene expression was measured by qRT-PCR in a cohort of 144 cancer tissues and 6 normal control samples. Median expression in cancer tissues was 1.8-fold higher than in normal controls (Figure 4a, Mann–Whitney *U* test; $p = .142$). Expression of six outliers was even
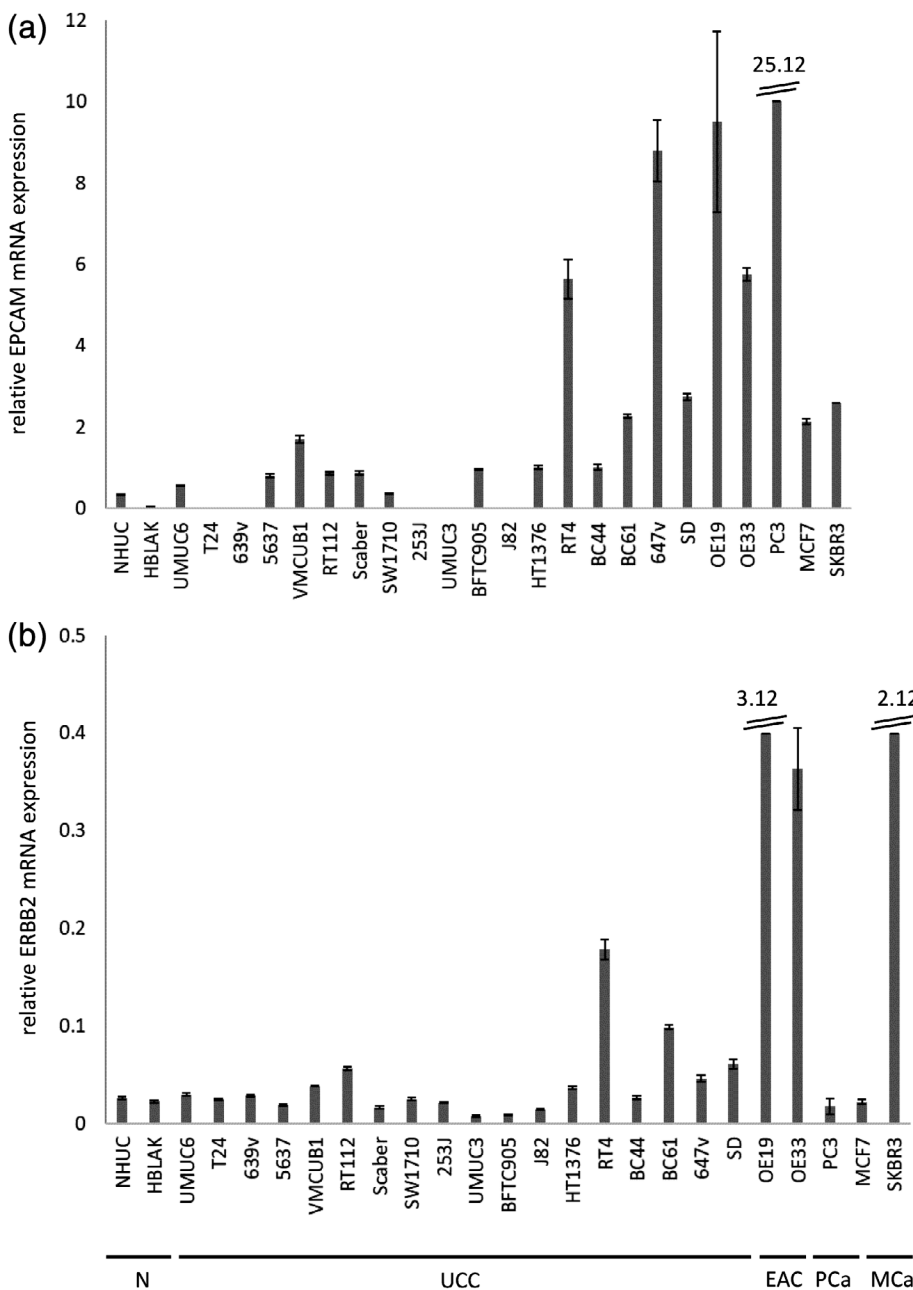
FIGURE 1 *EpCAM* und *ERBB2* mRNA expression in UCC lines and control cell lines. Expression of *EpCAM* (a) and *ERBB2* (b) mRNA was measured by qRT-PCR in UCC lines and in the immortalized non-neoplastic urothelial cell lines HBLAK and NHUC-TERT (N). Further control cell lines from other cancer entities were included. Expression was adjusted to *TBP* mRNA levels as a reference gene. EpCAM, epithelial cell adhesion molecule; EAC: esophageal adenocarcinoma, MCa: mammary breast cancer; PCa: Prostate cancer; UCC, UC cell lines

much stronger increased, up to 65-fold higher than median expression of control samples, which may result from *ERBB2* amplifications. *ERBB2* expression did not correlate with patients' characteristics (Table 2); however, Kaplan–Meier analysis revealed that *ERBB2* expression above median in cancer tissues across the complete patient cohort was significantly associated with metastasis free survival (Figure 4b; Log Rank Mantel-Cox: 0.033). When we divided the cohort between patients <pT2 and those ≥pT2 significance was even further increased (Figure 4c; Log Rank Mantel-Cox: 0.013) demonstrating that HER2 is more strongly increased in patients with muscle-invasive bladder cancer and particularly associated with risk of metastasis. This correlation remained also significant in the multivariate analysis (Figure 4c, hazard ratio 2.632 (95% confidence interval: 1.026–6.750, p = .044). These results indicate that HER2 is stably

expressed also during cancer progression and metastasis in UC and may thus be a more suitable CTC detection marker than EpCAM, which varies more strongly depending on the phenotype of cancer cells.

3.3 | Detection of HER2^pos cells by CellSearch CTC scan

To further analyze whether HER2 could be a suitable marker for CTC detection, we used HER2-positive UCCs for CellSearch CTC analysis. Therefore, healthy donor blood samples were spiked with UCC lines exhibiting significant EpCAM expression and varying quantities of HER2-positive cells, namely RT112 (99.5% HER2^pos), 5637 (60%

TABLE 4 Results of FACS analyses for protein expression of HER2 and EpCAM in UCC lines compared to the BC cell line SKBR3 used as a positive control for HER2

Cell line	Percentage of HER2+ cells (%)	RFMI HER2	Percentage of EpCAM+ cells (%)	RFMI EpCAM
T24 (M)	23.7	1.7	1.9	1.0
639v (M)	45	2.5	0.3	1.0
5637 (E)	74.0	3.6	99.1	16.1
VMCUB1 (E)	29.6	2.5	96.8	21.4
RT112 (E)	99.5	15.7	94.9	11.7
SW1710 (E/M)	66.7	3.8	94.7	7.5
253J (M)	32.0	2.5	0.4	1.0
UMUC3 (M)	18.9	1.8	1.1	0.9
BFTC905 (E)	93.4	8.3	98.7	28.6
J82 (M)	83	3.5	3.4	1.1
HT1376 (E)	32.0	3.5	97.8	19.7
SKBR3	98.5	35.5	—	—

Abbreviations: E, epithelial phenotype; EpCAM, epithelial cell adhesion molecule; M, mesenchymal phenotype; HER2, human epidermal growth factor receptor 2.
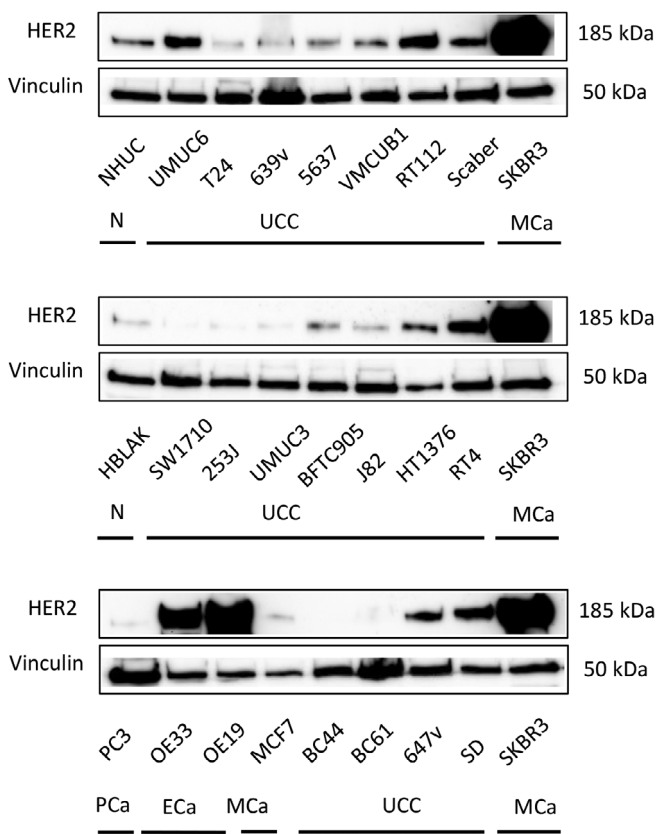

FIGURE 2 HER2 protein expression in UCC lines and control cell lines. Western blot analysis of HER2 expression in UCC lines (UCC) compared to benign control cell lines HBLAK and NHUC-TERT (N). Further control cell lines from other cancer entities were included. The MCa cell line SKBR3 with HER2 amplification was used as a positive control on each membrane. Vinculin was detected as a loading control. EpCAM, epithelial cell adhesion molecule; EAC: esophageal adenocarcinoma, MCa: mammary breast cancer; PCa: Prostate cancer; UCC, UC cell lines

HER2^{pos}) and VMCUB1 (25% HER2^{pos}, Table 5). SKBR3 cells were used as a control, as reported previously (Schneck et al., 2015).

Upon optical estimation of HER2 staining on the CellTracks Analyser II, SKBR3 was graded class 3+, whereas all analyzed UC cell lines were graded as class 1+ (Table 5 and Figure 5a). SKBR3 cells were HER2-positive with an intensity range of 100–600, whereas HER2 intensity ranged between 15 and 35 for UCCs. The SKBR3 cell line yielded 258/1000 EpCAM^{pos} cells of which 253 were HER2-positive (Table 5). By comparison, of 1,000 spiked RT112 cells 186 were detected by CellSearch of which 15 (8%) were HER2^{pos}; the numbers for 5637 cells are 731/1000 EpCAM^{pos} cells, of which 27 (4%) were HER2^{pos}, and the VMCUB1 cell line had 433/1000 EpCAM^{pos} cells, of which 12 (3%) were HER2^{pos}.

3.4 | HER2 expression and detection of HER2^{pos} CTCs in UC patients

Although the impact on patients' survival is discussed rather controversial, reports on positive HER2 expression as a prognosticator for the metastatic potential in UC patients are rather consistent (Fleischmann et al., 2011; Nedjadi et al., 2016). However, as HER2 expression in UC of the primary tumor and the metastases may differ significantly (Fleischmann et al., 2011) (Table 1), assessing HER2 status in CTCs would provide substantial information for assessing the patient's individual risk.

A previous study found only 3 of 16 patients with EpCAM^{pos} CTCs who were also HER2^{pos} (Nedjadi et al., 2016). These data raise the question whether (a) HER2 is really suitable for CTC detection and (b) whether HER2 expression in CTCs is reliable. We therefore investigated this issue in an independent sample cohort. Additionally, we explored how CTC numbers detected via EpCAM or HER2 change in consecutive samples in patients undergoing systemic therapy. For this purpose, 21 blood samples from 13 patients with metastatic UC
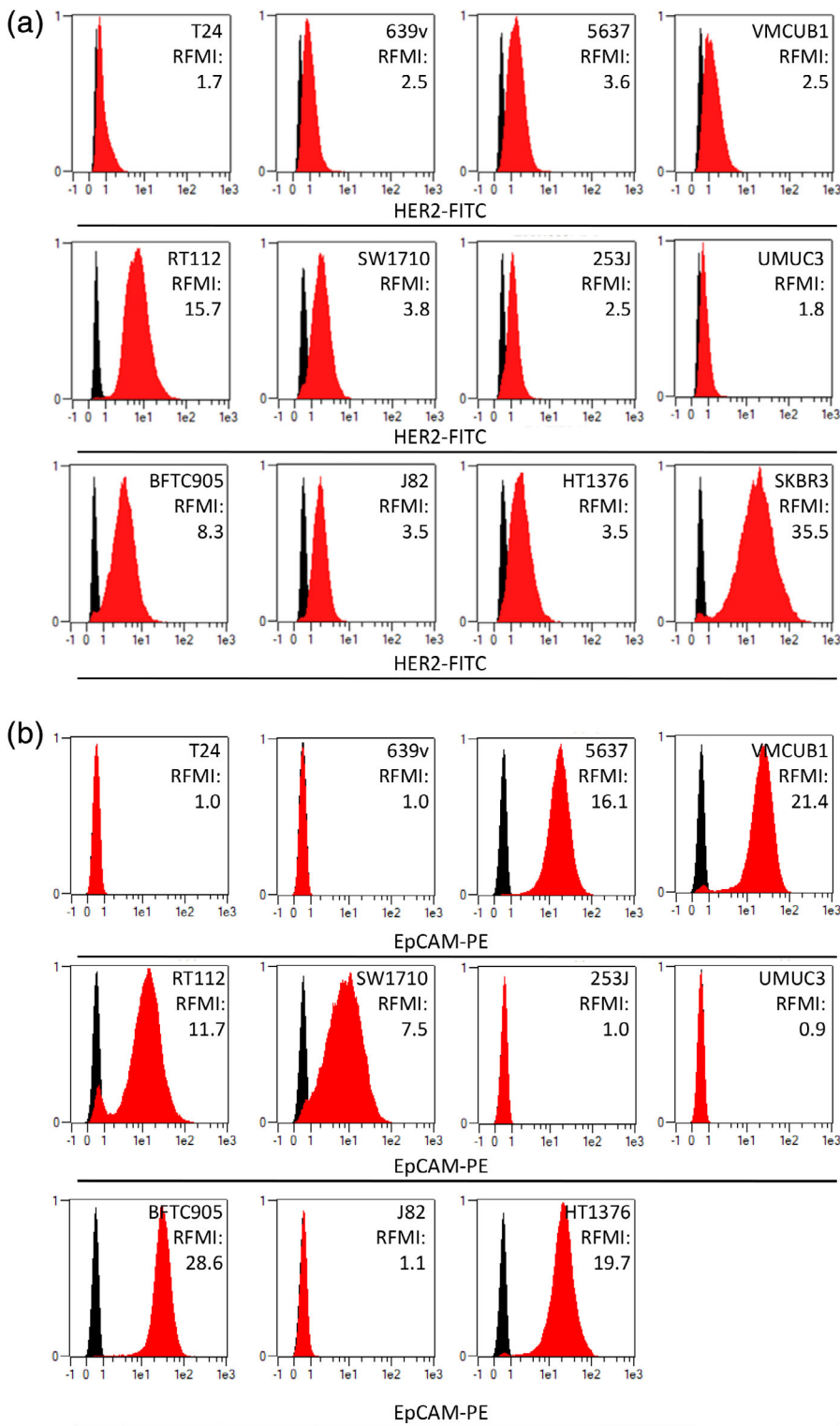
FIGURE 3 Flow cytometry data on protein expression of HER2 and EPCAM in UCC lines. Expression of HER2 (a) and of EpCAM (b) was dertermined by antibody staining and flow cytometry. SKBR3 cells served as a positive control for HER2 staining. Specific staining signals of HER2 protein expression (red peak) were overlayed on background expression of controls (black peak). The median fluorescence (RFMI) was calculated according to Siddiqui et al. (Siddiqui et al., 2019) as median fluorescence intensity HER2/median fluorescence intensity control. EpCAM, epithelial cell adhesion molecule; HER2, human epidermal growth factor receptor 2 [Color figure can be viewed at wileyonlinelibrary.com]

at diagnosis, relapse after surgery or after previous chemotherapy were tested (Table 1).

Via EpCAM selection, a positive enumeration (≥ 1 CTC) was observed in 7 of 21 (33%) samples and 6 of 13 patients. However, HER2^{pos} CTCs were only detected in one patient. Of this patient, a cluster of four CTCs was categorized as HER2 class 1+ in intensity (Figure 5b). In three of four patients with at least two consecutive

blood samples collected during systemic therapy a positive enumeration (≥ 1CTC) was observed at baseline. In each case, CTCs number decreased during chemotherapy. In 9 of 13 and 4 of 13 patients tissue samples of primary tumors and metastases before start of systemic therapy, respectively, were available for HER2 IHC scoring. HER2 IHC was positive (IHC score 3+) on 2 of 9 primary tumors and 1 of 4 metastasis. No or only weak HER2 expression (IHC 1+) was observed
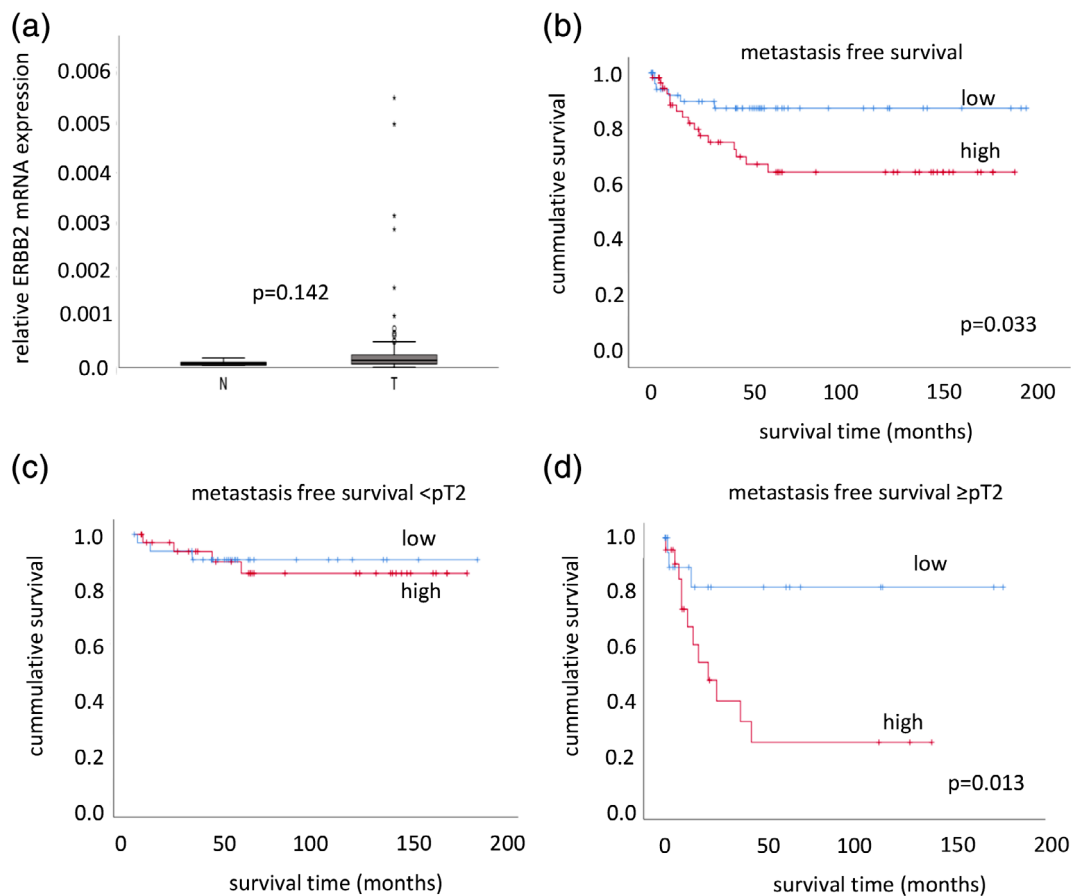
		Metastasis-free survival	
Variables	HR	95% CI	p
All cases (Ta-T4)			
Sex (male)	0.585	0.235 - 1.458	0.250
Stage (T2-T4)	2.971	0.847 - 10.413	0.089
Grade (G3)	2.725	0.778 - 9.542	0.117
LN / M status (N+ or M+)	-	-	-
HER2 (> median)	2.632	1.026 - 6.750	**0.044**

FIGURE 4   *ERBB2* mRNA expression and survival analysis for patients with UC. (a) Expression of *ERBB2* mRNA was measured by qRT-PCR in 144 cancerous patient tissues and normal controls. (b) Kaplan–Meier analysis was performed to illustrate the significant correlation between high *ERBB2* expression and poor prognosis regarding metastasis free survival for the complete cohort. (c) This relation became even more significant when the cohort was divided into patients with <pT2 (left panel) and ≥ pT2 (right panel). Also multivariate analysis confirmed significance (bottom panel). HER2, human epidermal growth factor receptor 2 [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 5   Detailed results of spiking experiments (cell numbers according to optical evaluation or ACCEPT evaluation)

	CTC HER2+ events (number of HER2+ EpCAM+ cells/absolute number of EpCAM+)	CTC HER2 intensity	ACCEPT HER2 intensity	ACCEPT CKs intensity
SKBR3	253/258 (98%)	3+	100–600	3,000–4,000
RT112	15/186 (8%)	1+	10–35	3,000–4,000
5637	27/731 (4%)	1+	15–35	1,000–4,000
VMCUB1	12/433 (3%)	1+	15–35	1,000–4,000

Abbreviations: ACCEPT, Automated CTC Classification Enumeration and PhenoTyping; CTC, circulating tumor cells; HER2, human epidermal growth factor receptor 2.
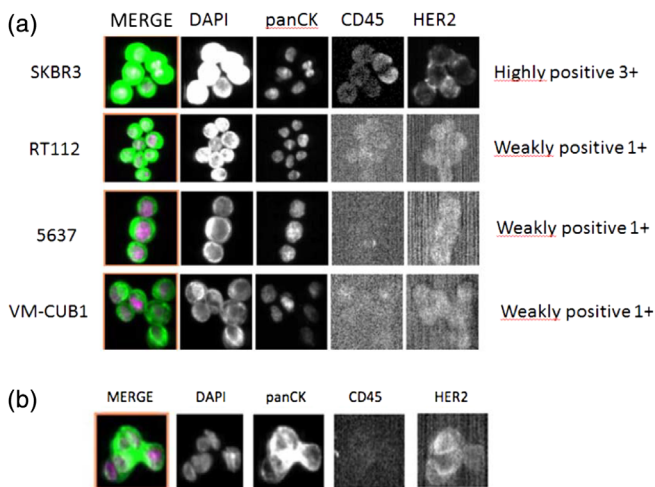
FIGURE 5 Optical estimation of HER2 staining in spike experiments. (a) Healthy donor blood samples were spiked with UCC lines (RT112, 5637, VM-CUB1) with significant EpCAM expression and varying quantities of HER2-positive cells (high, intermediate, and low, respectively) according to FACS analyses. SKBR3 cells were used as a positive control and reference for HER2 staining intensity (Nielsen et al., 2009). Epithelial cells captured by anti-EpCAM antibody coated ferrofluid were stained for cytokeratins (panCK) and HER2. An anti-CD45 antibody was used to exclude leukocytes. Nuclei were counterstained with DAPI. After enrichment cells were scanned using the CellTracks Analyzer II (Veridex) for optical estimation of HER2 staining; SKBR3 was graded class 3+, whereas all analyzed UC cell lines were graded as class 1+. (b) HER2 1+ positive cell cluster in a patient with metastatic UC. Blood samples from patients with metastatic UC were used for CellSearch CTC analysis. Epithelial cells captured by anti-EpCAM antibodies were stained for cytokeratins (panCK) and HER2. An anti-CD45 antibody was used to exclude leukocytes. Nuclei were counterstained with DAPI. After enrichment, cells were scanned using the CellTracks Analyzer II (Veridex) for optical estimation of HER2 staining. EpCAM-enriched CTCs stained positive for HER2 as well only in one patient, these were categorized as class 1+ . EpCAM, epithelial cell adhesion molecule; HER2, human epidermal growth factor receptor 2; UCC, UC cell lines [Color figure can be viewed at wileyonlinelibrary.com]

in 4 of 9 primary tumors and 3 of 4 metastases. Except for one case, in which a hepatic metastasis with a weak HER2 expression (IHC score 1+) was observed in a patient with no HER2 expression (IHC score 0) in the primary tumor, HER2 expression of primaries und metastases corresponded well in our patient cohort. Of note, though a high number of CTCs (78 and 30) was observed in both patients with HER2 positive IHC, no HER2 positive CTCs were detected. In contrast, IHC scoring for primary tumor and metastasis was negative in the patient, in which HER2 positive CTCs were detected (primary tumor: IHC score 0, hepatic metastasis: IHC score 1+).

4 | DISCUSSION

Several observations of the present study are of importance. First, *ERBB2* mRNA expression was comprehensively investigated in 18

commercially available UCC lines representing the whole clinical width of the disease's heterogeneity regarding tissue of origin and cellular phenotype. *ERBB2* expression was compared with benign controls and cells lines from other cancer entities both with and without HER2 gene amplifications. *ERBB2* expression could be detected in all investigated UCC lines. Variation in its expression showed no clear relation to gender, differentiation grade, histology or stage of the cancer of origin proving HER2 to be a more robust biomarker than EpCAM. Data published by Hayashi et al. (Hayashi et al., 2015) also support our observation that *ERBB2* expression in UCC is not strongly dependent on cellular phenotype. While EpCAM expression may be downregulated during EMT, strong HER2 overexpression is reported to induce EMT (Ingthorsson et al., 2016; Nami & Wang, 2017). Thus, high HER2 expression is maintained in EMT-positive metastasizing tumor cells. Accordingly, based on our data and the data from the literature, that addition of an antibody directed against HER2 to an EpCAM-based enrichment might be beneficial in bladder cancer CTCs prone to undergo EMT. In fact, HER-based immunomagentic enrichment of tumor cells, for example, from blood or bone marrow has been described before (Jain, Veggiani, & Howarth, 2013; Wang et al., 2018; Woelfle et al., 2005).

The CCLE database comprises 20 cell lines originating from urinary tract, of which 10 were included in our set of 18 UCC. In contrast to the BC cell line SKBR3 and esophageal cell lines, no UCC line is described to carrying any *ERBB2* copy number changes in the CCLE database which is concurring with robust expression among the UCC set but is magnitudes lower than in HER2 amplified cancers. Likewise, only few commercially available UCC have been reported to carry *ERBB2* mutations. De Martino et al. (de Martino et al., 2014) only detected mutations in 5 of 33 UCC (VMCUB3, DSH1, 5637, VMCUB1, J82). Two cell lines carried the activating S310F mutation, other detected mutations were not associated with gain of function. Open databases report three *ERBB2* missense mutations in the UCC lines used in our investigation (p.R678Q for J82, p.S310F for 5637, p. S653C for VMCUB1) and two silent mutations (p.Q646Q for VMCUB1 and p.V669V for 647-V) (https://cancer.sanger.ac.uk/cell_lines). Thus, only a few UCC lines corresponding to Clusters I and II of the TCGA cohort (e.g., 5,637 and J82) harbor mutant *ERBB2* and provide useful models for targeted therapy.

Clinical relevance for UC patient tissues was reported by previous studies demonstrating that *ERBB2* mRNA levels were upregulated both in nonmuscle invasive and muscle invasive UC (Junttila et al., 2003). Instead, *ERBB2* mutations and amplification were enriched among specific UC subtypes, that is, Cluster I and II in the 2014 TCGA cohort (Cancer Genome Atlas Research N, 2014). Overall, 9% of the tumors from that study were considered as potentially sensitive to HER2 kinase inhibitors or antibodies due to mutations or amplifications of the gene (Cancer Genome Atlas Research N, 2014). In the updated 2017 TCGA study of muscle-invasive UC, the percentage of genetically altered samples rose to 17%, with one recurrent S310F mutation in the extracellular domain accounting for 42% of the mutant cases (Robertson et al., 2017). Concurringly, we observed *ERBB2* overexpression in UC tissues compared to normal controls.

Some outlier cancer samples stood out with extraordinary high *ERBB2* expression, probably due to genetic changes. Importantly, in our cohort increased *ERBB2* expression was even more significantly associated with risk of metastasis in muscle-invasive cancers compared to patients with non-muscle-invasive cancers. Likewise, Kriegmair et al. (Kriegmair et al., 2018) analyzed a cohort of muscle-invasive UC and reported about poor prognosis for patients with high *ERBB2* expression. Thus, in contrast to *EPCAM*, *ERBB2* expression appears to be more independent of cellular morphology and phenotypic changes that may occur during cancer progression and metastasis, for example, EMT. *ERBB2* may even be more strongly expressed in advanced cases increasing the probability that HER2 may be detectable on CTCs of corresponding cases.

Second, HER2 protein expression was likewise detectable in all UCC lines and its variation too was not strongly associated with particular cell line features. In FACS, the fraction of HER2-expressing cells was highly variable, although the intensity of the signal, which should parallel the amount of HER2 on the cell surface, was usually higher in cells with a more epithelial morphology. Importantly, the expression of HER2 protein in SKBR3 cells, which are an established model for BC with an *ERBB2* gene amplification, was orders of magnitudes stronger. Similar results were reported by Siddiqui et al. (Siddiqui et al., 2019) who investigated HER2 expression on UCC by FACS to identify models for development of a photo-immunotherapy based on EGFR and HER2 expression. Of our 11 cell lines investigated by FACS, five cell lines were also analyzed by Siddiqui et al. also demonstrating detectable HER2 expression across UCC, but magnitudes lower than in SKBR3. Accordingly, in our spike-in experiments for CTC detection, UCC lines were categorized as 1+ as compared to 3+ for SKBR3. This finding does not bode well for the use of HER2 as a marker for UC-CTCs. Indeed, Rink et al. (Rink et al., 2012) found that only 3 of 16 patients with EpCAM^pos CTCs were HER2^pos as well. Accordingly, in our analysis of CTCs from patients with metastatic UC, HER2^pos CTCs could only be detected in one case, with relatively weak staining (1+). Several authors have drawn attention to similarities between UC Clusters I and II and BC subtypes, especially with regard to HER2. Our data suggest, however, that although some UCs express HER2, its extent of expression is not comparable to that in BC belonging to the HER2+ subtype. Instead, differences in HER2 expression in UC molecular subtypes may to some extent reflect the higher expression of HER2 in the upper layers of the urothelium, whereas EGFR is more strongly expressed in basal cells (Eriksson et al., 2017).

Third, UCs cell lines with a mesenchymal phenotype had a much lower expression of *EPCAM* mRNA and protein than those with an epithelial phenotype. One exception was the SW1710 cell line, which indeed has an intermediate E/M phenotype. Assuming that partly or fully mesenchymal cells constitute some of the CTCs in UC patients, this finding explains to some extent that only 7 of 21 samples in our cohort of metastatic patients had EPCAM^pos CTCs. It has recently been observed in both the TGCA and the Lund classification systems (Seiler et al., 2017) that UCs with an EMT signature tend to have a worse prognosis. Our findings accord with previous observations,

where only two (RT4 and T24) out of five UC cell lines (UMUC3, 253J and TCCSUP) yielded positive signals for EpCAM in spike-in experiments (Okegawa, Hayashi, Hara, Nutahara, & Higashihara, 2010). An immunohistochemical staining for EpCAM in primary and metastatic UC specimens demonstrated that UC has the highest negative (56%) and weak staining rate (17%) among various human cancers (Fong et al., 2014). This low rate may partly derive from EMT, during which EpCAM is downregulated (Gao, Yan, Wang, Liu, & Yang, 2015; Junttila et al., 2003; Liu et al., 2014; Santisteban et al., 2009; Ye et al., 2015), as corroborated in our cell line series. Therefore, the relatively low rate of CTC detection in metastatic patients in the present study may be linked to EMT of the tumor cells, with reduced expression of EpCAM. Furthermore, EpCAM expression on the cell surface may be limited by shedding. Thus, UC patients with high levels of urinary truncated EpCAM had a worse prognosis compared to other patients with the same pT stage, but low or negative levels of urinary EpCAM (Bryan et al., 2015). In summary, although technically feasible, the use of EpCAM as a "biologic hook" to catch CTCs in UC patients may have limited utility and very likely leaves a substantial fraction of cancer cells in the blood undetected. Our study, however, also demonstrates that detection of CTCs via HER2 will not yield improvements, despite its broad expression.

Finally, our study also has implications for the identification of HER2 UC subgroups for targeted therapies (Hayashi et al., 2015). The use of lapatinib as a maintenance regime in patients with stable HER1/HER2 positive metastatic urothelial carcinoma (mUC) after first-line chemotherapy failed to show any difference in survival (Powles et al., 2017). One reason for this failure may be that protocols to assess HER2 status may need to be adapted specifically for use in UC (Kiss et al., 2017; Rink et al., 2012) which may also explain why studies disagree on the prognostic role of HER2 overexpression (Bolenz et al., 2010; Kassouf et al., 2008; Kruger et al., 2002; Soria et al., 2016) (Cancer Genome Atlas Research N, 2014; de Martino et al., 2014; Junttila et al., 2003; Kriegmair et al., 2018). Our findings clearly indicate that analysis of CTCs for HER2 expression does not offer a feasible alternative, at least not with current techniques. Whether other CTC marker-dependent systems (e.g., CTC-iChip technology) or marker-independent enrichment strategies, for example, using microfluidic systems (e.g., Parsortix or Clearbridge microfluid systems), will lead to improved stratification of UC patients for HER2-targeted therapies needs to be further explored (Karabacak et al., 2014) (Siddiqui et al., 2019). Using cell-independent detection systems may be another alternative for assessing the HER2 status of urothelial carcinoma patients, by, for example, analysis of cell free DNA (cfDNA). Recent studies pointed out that this kind of analysis might be possible both in urine and blood samples (Christensen et al., 2019; Lee et al., 2018).

5 | CONCLUSIONS

In summary, our data show that HER2 is expressed in UCC lines independent of the cell lines' phenotypes (epithelial vs. mesenchymal) but

varies in intensity. However, even in UCC lines containing a high fraction of HER2^{pos} cells, HER2 expression was only of low intensity when compared to the HER2^{pos} BC cell line SKBR3. The relatively weak intensity of HER2^{pos} cells (at least in EpCAM captured UCC) is the most likely factor impairing detection by CellSearch analysis.

Our results were confirmed by our analysis of a cohort of patients with metastatic UC. It is remarkable that EpCAM[pos] CTCs were detected only in 6 of 13 patients despite the confirmed presence of metastatic disease. However, EpCAM/HER2-double positive CTCs were detected in only one of these patients.

Therefore, we conclude that current EpCAM-based protocols for the detection of CTC in UC patients are not optimal. In particular, they cannot be relied on to assess HER2 status of patients suffering from advanced UC or to enrich CTC by HER2 as (a) they will miss EpCAMlow/[neg] but HER2^{pos} CTCs and (b) they may possibly miss a significant number of EpCAM[pos] HER^{pos} cells as well.

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