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ORIGINAL ARTICLE

HER2 and TOP2A Gene Amplification and Protein Expression in Upper Tract Urothelial Carcinomas

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Abstract *HER2*, a potential target for therapy, has been described to be amplified in urothelial carcinomas. As the topoisomerase II alpha (*TOP2A*) gene is located close to the *HER2* gene on chromosome 17q12-q21, it is frequently either co-amplified or deleted with *HER2* amplification. The purpose of this study was to assess the impact *HER2* and *TOP2A* gene amplification as well as protein expression on outcomes of upper tract urothelial carcinoma (UTUC). *HER2* and *TOP2A* gene amplification and protein expression were assessed in 81 patients with radical nephroureterectomy for UTUC. Immunohistochemistry and chromogenic in-situ hybridization was performed on formalin-fixed, paraffin-embedded samples. HER2 protein expression was observed in 27/81 (33%) cases, of which 8 cases exhibited amplification of *HER2*. One of them had an additional polysomy 17, whereas 6/67 HER2 non-amplified cases revealed a polysomy 17. Coamplification of *HER2* and *TOP2A* was found in 4 cases, whereas 3 cases showed only *HER2* amplification and 20 cases only *TOP2A* amplification. HER2 IHC overexpression was associated with higher-grade tumors *($p = 0.001$)*, non-organ confined carcinomas *($p = 0.017$)*, HER2 amplification *($p < 0.00001$)* and TOP2A amplification *($p = 0.016$)*. HER2 amplification was association with higher tumor grade *($p = 0.001$)* and lymphnode metastasis *($p = 0.003$)*. TOP2A IHC positivity was significantly associated with higher tumor grade *($p = 0.0004$)*, TOP2A amplification *($p = 0.0003$),* polysomy 17 *($p = 0.035$)* and HER2 IHC overexpression *($p = 0.28$)*, whereas all categories of tumor stage and HER2 amplification remained not related. TOP2A amplification was significantly more frequent in tumors with higher grade, higher tumor stage, polysomy 17 and distant metastasis *($p = 0.015$; $p = 0.042$; $p = 0.032$; $p = 0.011$), respectively*. In univariate analyses HER2 IHC positivity, TOP2A amplification, and polysomy 17 were associated with poor clinical outcome after surgery. HER2 IHC overexpression and TOP2A amplification are associated with features of biologically aggressive UTUC. Overexpression and/or amplification of *HER2* and *TOP2A* could help identify patients who may benefit from targeted therapy.

Keywords HER2 · TOP2A · Polysomy 17 · Amplification · Anthracyclines · Urothelial carcinoma

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Introduction

HER2 expression is associated of features of advance disease and poor prognosis in various malignancies [1]. Targeting HER2 with monoclonal antibodies has resulted in significant reduction of tumor recurrence and death in breast cancer [2–5]. In urothelial carcinomas HER2 is expressed in approximately 5–15% of tumors [6] and seems to predict worse pathology and outcomes when overexpressed in bladder cancer histologic specimen [7] as well as circulating tumor cells in peripheral blood [8].

In upper tract carcinoma (UTUC), data on the prognostic value of HER2 is limited, moreover, most studies used IHC to assess HER2 protein expression, while gene amplification may be more reliable to assess HER2 status [9–14].

As the *topoisomerase II alpha* (*TOP2A*) gene is located close to the *HER2* gene on chromosome *17q12-q21*, it is frequently either co-amplified or deleted in tumors with *HER2* amplification [15]. TOP2A is a DNA-modifying enzyme that binds to the double helix to release torsional stress and create doublestrand breaks that promotes replication and therefore is responsible for DNA metabolism (transcription, recombination, replication, chromosomal segregation) during the cell division [16]. TOP2A alterations may therefore prognosticate outcomes and/or predict response to anthracycline-based combination chemotherapy [9–12]. The prognostic value of TOP2A has so far never been investigated in UTUC.

The purpose of this study was to evaluate *HER2* and *TOP2A* gene amplification and protein status in UTUC patients treated with radical radical nephroureterectomy (RNU).

We hypothesized that HER2 and TOP2A expression is associated with worse outcomes in UTUC patients.

Material and Methods

Case Selection

Formalin-fixed and paraffin-embedded specimens of 124 patients with UTUC treated with RNU between 1986 and 2002 of a single center were included into this retrospective analysis. The study was conducted following the rules of ICH-Guideline for Good Clinical Practice and the ethical principles for medical research according to the declaration of Helsinki. The use of human material for the analysis was approved by the local ethical committee [13]. Well-documented follow up was available of 81 individuals. Disease free survival was calculated from time of primary surgery until first evidence of progression of disease. Survivals until end of observation period or losses to follow up were considered as censored observations. Tumor grading and staging was performed according to the UICC guidelines [17].

Immunohistochemistry of HER2

All tumor specimens were fixed in 7.5% formalin overnight, stained with hematoxylin and eosin (HE). All HE stained slides were reviewed by two of the authors (KA, AH).

Immunohistochemistry for HER2 protein expression was carried out using the HercepTest (Dako, Glostrup, Denmark), according to the manufacturer's instructions. The primary polyclonal antibody against rabbit anti-human HER2 oncoprotein (A0485; DAKO) was applied followed by incubation for 30 min at room temperature. After washing, the specimens were incubated with visualization reagent (provided) and applied with DAB substrate chromogen. Counterstaining was carried out with hematoxylin (Fig. 1).

The expression of HER2 was classified as described previously [18]. Briefly, we referred no reactivity or membranous reactivity in <10% of cells as negative (0), faint or barely perceptible membranous reactivity in ≥10% of cells or cells are reactive only in a part of their membrane as negative (1+), weakly positive to moderate continuous expression through the entire cell membrane or lateral/basolateral membranous reactivity in ≥10% of tumor cells as equivocal (2+) and strong continuous expression through the entire cell membrane or lateral/basolateral membranous reactivity in ≥10% of tumor cells as positive (3+).

Immunohistochemistry of TOP2A

After deparaffinization and rehydration, endogenous peroxidase activity was blocked by incubation with methanol/H2O2 mixture. The tissue sections were micowaved in citrat buffer, then blocked by horse serum for 15 min. The primary polyclonal antibody Topoisomerase II α AK (Mouse anti Topo II α, 2nd Predilute Antibody, clone 3F6, Zymed, ready to use) against TOP2A was applied followed by incubation for 30 min at room temperature. Detection was accomplished by incubation of biotinylated secondary antibody, followed by the tertiary ABC complex, which bound to biotin on the secondary antibody. Slides were then developed with an AEC Substrate Kit (Zymed Laboratories Inc., San Francisco, CA). Sections were counterstained with hematoxylin. For all immunohistochemical stainings controls were performed by omitting the first antibody. For interpretation of the immunoreactivity of TOP2A only nuclear staining was rated positive. We counted the percentage of positive nuclei by counting 1000 cells independently of intensity within hot spots using an integration grid.

Chromogenic In-Situ Hybridization of HER2 and TOP2A

CISH for detection of HER2 was done on 3 mm-thick histological sections. Before hybridization, tissue sections were deparaffinized in xylene, washed in 100% ethanol, and air-

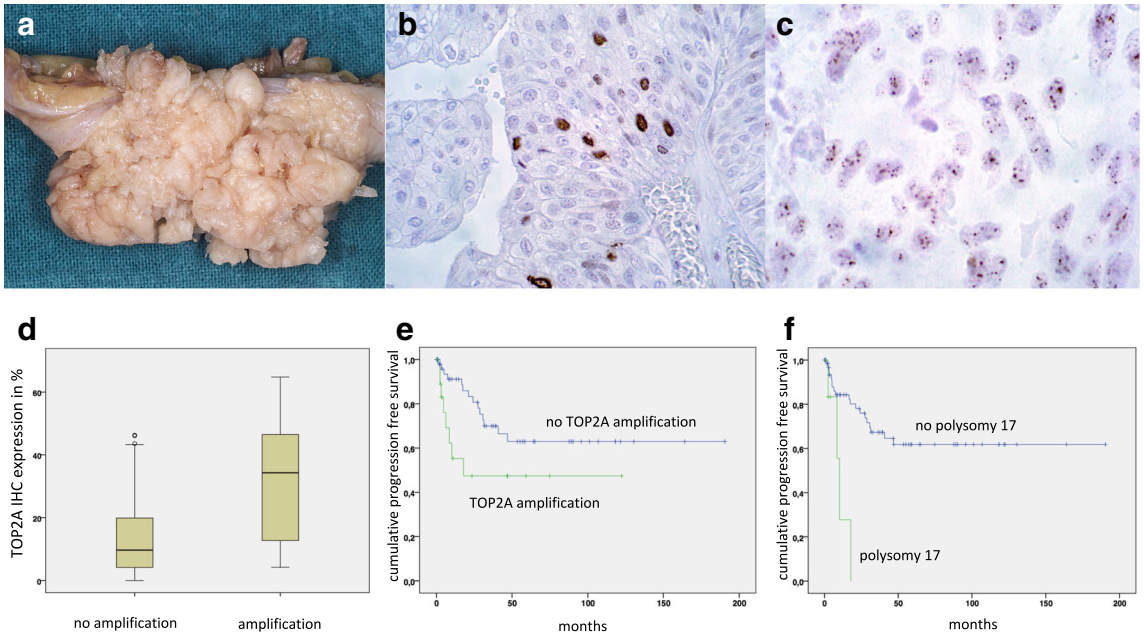

Fig. 1 (**a**) Upper tract urothelial carcinoma (UTUC), pT1, G2 (**b**) TOP2A immunohistohemistry, x400 (**c**) Scattered clusters of amplified TOP2A, x400 (**d**) Tumors with TOP2A amplification showed higher TOP2A expression levels (p=0.0003) (**e**) Univariate survival analysis of TOP2A amplification (p=0.05) (**f**) univariate survival analysis of polysomy 17 (p=0.001)

dried. Slides were then incubated in 0.1 M Tris-saline (pH 7.0) at 98–100 °C in an autoclave for 15 min at 1 bar. After cooling at room temperature for 15 min and rinsing twice in PBS for 3 min, the tissue sections were covered with 200 ml of pepsin solution (Digest-All 3; Zymed, South San Francisco, California) at 37 °C for 6 to 8 min. The slides were then washed in PBS three times at room temperature for 2 min, dehydrated in graded ethanols, and air-dried. The ready-to-use digoxigenin- labeled *HER2* probe (Zymed, Spot Light Amplification Kit) was applied to the tissue sections. Slides were coverslipped and sealed with rubber cement to prevent evaporation of the probe solution. Slides and the probe mixture were denaturized at 94 °C for 3 min on a thermal plate, and hybridization was carried out in a humid chamber at 37 °C overnight. After hybridization, the slides were washed with 0.5× SSC for 5 min at 80 °C, followed by three washes in PBS for 2 min at room temperature. The HER2 probe was detected with sequential incubations with Blocking Reagent (Reagent A), Anti-Mouse anti DIG antibody (Reagent B), Polymerized HRP-anti-mouse (Reagent C) (Zymed, CISH Polymer Detection Kit) and DAB (ImmunoVision Technologies) as chromogens. After counterstaining with hematoxylin, the slides were dehydrated and embedded.

Each tumor was also classified by chromosome 17 (Ch17) copy number status (Zymed, CISH Polymer Detection and Centromere Detection Kit).

CISH with *TOP2A* probe was technically performed by the same method, however using the Spot-Light *Topoisomerase II alpha* probe (Zymed, South San Francisco, California). For interpretation of HER2 as well as TOP2A amplification the manufacturer's manual (Ventana Medical Systems) was used.

Statistical Analysis

Pearson's chi-square tests, and Mann-Whitney *U* tests were used as appropriate. Kaplan-Meier estimates and log-rank tests were used for univariate analysis of survival. Multivariate analysis of survival was performed using the Cox proportional hazard model in a backward manner. A *p*-value of ≤0.05 was considered to be statistically significant. SPSS 20.0 (IBM, Armonk, NY) was used for all calculations.

Results

Characterization of Patients

The median age of patients was 68,0 years (mean 66.3 years, range 39 to 92 years). 37 patients were female, 44 male. The median follow-up period was 17.4 months (mean 35.1 months, range 0.1–190.5 months). 23 patients (28%) experienced disease recurrence. None of the patients received neoadjuvant or adjuvant treatment after operation surgery. Detailed results are given in Table 1.

HER2

HER2 protein expression was observed in 27/81 cases. HER2 IHC positivity was associated with HER2 amplification ($p < 0.00001$). HER2 overexpression was associated with higher-grade tumors ($p = 0.001$). *HER2* amplification was found in 8 cases (10%); one of them showed an additional

polysomy 17. *HER2* amplification was significantly more often found in higher-grade tumors ($p = 0.001$) and pN1 patients ($p = 0.003$), but not with TOP2A amplification, tumor stage, distant metastasis, and polysomy 17. Coamplification of *HER2* and *TOP2A* was found in four cases, whereas three cases showed only *HER2* amplification and 20 only *TOP2A* amplification.

TOP2A

Variable positive expression of TOP2A protein could be counted in all 81 cases; the mean/median percentage of stained cells was 41% (1–81%). Immunohistochemical TOP2A expression rate was significantly associated with higher tumor grade *($p = 0.0004$)*, TOP2A amplification *($p = 0.0003$)* polysomy 17 *($p = 0.035$)* and HER2 IHC overexpression *($p = 0.28$)*, whereas all categories of tumor stage and HER2 amplification remained unrelated.

TOP2A amplification was revealed in 19/79 cases (24%) cases, 4 of them showed an additional polysomy 17. *TOP2A* amplification was significantly associated with tumor grade *($p = 0.015$)*, tumor stage *($p = 0.042$)*, polysomy 17 *($p = 0.032$)* as well as presence of distant metastasis *($p = 0.011$)*, but not lymph node metastasis.

Although, a cut off point analysis was performed, by intervals of 10%, to identify its stratifying cut off for TOP2A expression, no association between TOP2A expression and survival could be found.

TOP2A amplification was statistically more often found in HER2 IHC positive tumors compared to HER2 IHC negative tumors *($p = 0.016$)*. Polysomy 17 was found more often in tumor with TOP2A amplification compared to tumors without TOP2A amplification *($p = 0.032$)*. HER2 amplification and TOP2A amplification were not associated.

Table 1 Characterization of patients

	N (%)
Grading:	
G1	9 (11)
G2	35 (43)
G3	37 (46)
Staging:	
pTa	11 (14)
pT1	21 (26)
pT2	9 (11)
pT3	31 (38)
pT4	9 (11)
Lymphnode Status:	
N0	69 (85)
N1	12 (15)
Distant Metastasis:	
M0	75 (93)
M1	6 (7)
HER2 Immunostaining:	
0	54 (67)
1 +	13 (16)
2 +	9 (11)
3 +	5 (6)
HER2 CISH:	
No amplification	73 (90)
Amplification	8 (10)
TOP2A CISH:	
No amplification	60(76)
Amplification	19 (24)

Polysomy 17

Polysomy 17 was statistically more often found in high-grade tumors *($p = 0.026$)*, tumors of higher stage *($p = 0.044$),* and tumors with distant metastasis *($p = 0.025$)*. Polysomy 17 was not associated with lymphnode status.

Survival Analysis

In univariate analyses high tumor grade, higher tumor stage, distant metastasis, HER2 IHC, TOP2A amplification as well as polysomy 17, but not lymphnode metastasis, HER2 amplification or TOP2A IHC, were associated with tumor progression (Table 2).

In multivariate analysis including HER2 IHC and amplification as well as TOP2A IHC and amplification, polysomy 17, tumor grade and all categories of tumor stage, only distant metastasis and tumor stage (organ confined versus non organ confined) remained significant ($p = 0.0005$ *and* $p = 0.031$, respectively).

Discussion

This study investigates HER2 and for first time TOP2A protein overexpression and gene amplification in correlation to clinicopathologic data in UTUC. The incidence of HER2 positivity (3+ immunohistochemistry and/or amplification) of bladder cancer has been reported as many as 5 to 15% [6], the expression of HER2 protein from 9 to 81% [19]. In UUTUCC, only 7 reports concerning HER2 have been published so far. Here, HER2 positivity ranged from 0 to 53%. [9–14].

In our study, HER2 positivity was 10%, which is mostly comparable with the latest studies from Vershasselt-Crinquette et al. [20] 8%, Tsai et al. [21] 13.8%, and Bolenz et al. [7] 27.8%. The largest studies from Sasaki et al. [18] found a prevalence of 18% ($n = 171$). In general, older studies found higher prevalences. An explanation for higher prevalence in other studies could be related to different detection

Table 2 Progression-free survival analysis

	p-value
Grade (low grade vs high grade)	0.001
Stage pT (pTa vs pT1 vs pT2 vs pT3 vs pT4)	0.000005
Lymphnode metastasis pN	ns
Distant metastasis pM	0.000005
HER2 IHC (positive vs negative)	0.029
HER2 amplification	ns
TOP2A IHC	ns
TOP2A amplification	0.049
POLYSOMY 17	0.001

methods, lack of performing amplification, polysomy 17 controls and scoring systems. Our data showed that HER2 positivity correlated significantly with tumor grade and stage. These results are in concordance with all other authors with exception of a very small study from 1994 [14].

The authors Imai et al. [22], Langner et al. [23], Sasaki et al. [18] and Tsai et al. [21] reported in multivariate analysis that tumor stage and HER2 positivity were independent predictors of disease progression and overall survival. Interestingly, Tsai et al. [24] showed in another study that HER2 immunoreactivity had a limited prognostic value for advanced urothelial carcinoma patients with adjuvant MVEC (methotrexate, vinblastine, epirubicin, and cisplatin) therapy. In contrast, our study did not find HER2 to be an independent factor of progression. Probably, differences in the surveillance period, or the small size of the patient series, as well as the use of a tissue microarray may influence the variables of independence. Generally, results of HER2 positivity obtained in bladder cancer were comparable. [6, 19, 25–27].

As Sasaki et al. [18], we also used the modified scoring system by Hofmann et al. [28], which was originally developed for gastric cancer. This scoring system addresses both characteristics of HER2 protein expression (lateral/basolateral membranous reactivity and strong heterogeneity) appropriately and gave consistent results of immunohistochemistry compared to *HER2* gene status. The scoring system for breast cancer was not suitable for detection of HER2 status in UTUC due to the lack of complete membranous staining.

To date, only a few studies have reported the expression of TOP2A in bladder cancer [26, 29–31]. In all studies, high expression rates of TOP2A were found as well as an association with progress. TOP2A IHC expression has so far never been investigated in UTUC. In our setup, TOP2A IHC expression was associated with higher tumor grade, HER2 IHC overexpression, and polysomy 17 as well as TOP2A amplification. In addition, TOP2A amplification was associated with higher grade, stage (pT and pM), and polysomy 17.

Segmental ureteral resection with wide margins or radical nephroureterectomy with excision of the bladder cuff is the gold standard treatment for UTUC. Since UTUC are urothelial tumors, platinum-based chemotherapy is expected to produce similar results to those seen in bladder cancer. Randomized clinical trials comparing the outcomes of different treatments do not exist so far. Interestingly, adjuvant chemotherapy achieves a recurrence-free rate of up to 50% but has minimal impact on survival [32]. In our study, statistically significant overexpression of TOP2A was more often found in tumors with polysomy 17. According to these data, polysomy 17 may be used as an additional indicator for anthracycline-based (doxorubicin, epirubicin, etoposide) adjuvant chemotherapy sensitivity.

In breast cancer, recent studies suggest that the value of HER2 for predicting response to anthracycline- based chemotherapy may be more likely related to the concomitant amplification of the TOP2A gene. In our study, *HER2* was amplified in 8/81 (10%), *TOP2A* was amplified in 19/79 (24%) of the tumors of which 4 (5%) demonstrated coamplification. To define a subset of coamplified *HER2* and *TOP2A* in UTUC's that may reveal improved chemosensitivity, further studies with higher case numbers are needed.

Polysomy 17 was found in 7 cases (9%) and was significantly associated with tumor grade and stage in our data setup. Vershasselt-Crinquette [20] observed in 25% of the cases polysomy 17 and these also were found to correlate with tumor grade, tumor stage but without prognostic value. Langner [23] also found polysomy 17 or HER2 amplification to be significantly associated with tumor stage, grade and prognostic significance in univariate analysis. The impact of polysomy 17 alone in UTUC has not been well investigated. In bladder carcinoma, it has been found to be associated with muscle invasion and recurrence of the disease. [33] In our investigation polysomy 17 but not HER2 amplification are of prognostic value.

According to the EAU Guidelines for UTUC [32] to tumor stage and grade are the primary prognostic factors. Lymphovascular invasion but also necrosis (>10% of the tumor area), sessile growth pattern, microsatellite instabilities, and the molecular markers E-cadherin and hypoxia-inducible factor (HIF1a) are additional independent predictors of survival. Our data revealed TOP2A amplification as well as polysomy 17 and HER2 IHC to be prognostic markers associated with worse outcomes, however not independently. In multivariate survival analysis only tumor stage (pT and pM) remained as an independent prognostic predictor. Interestingly, in urothelial carcinomas of the bladder gene amplification of *HER2* and overexpression of HER2 protein have been identified as an independent predictor for disease-related survival and is associated with a more aggressive tumor phenotype and poor prognosis. [19, 34–37].

Conclusions

HER2 and TOP2A protein overexpression and/or gene amplification were found in high-grade upper urinary tract urothelial carcinomas. HER2 IHC positivity, TOP2A amplification and polysomy 17 predict poor outcome in univariate analysis and may serve as a target for Herceptin therapy and anthracycline-based adjuvant chemotherapy.

Author Contributions Information on how each individual author contributed to the original article:

1. Conception and design, or analysis and interpretation of data: Klaus Aumayr, Andrea Haitel.
2. Analysis of data: Barbara Neudert.
3. Drafting the article: Klaus Aumayr.
4. Revising it critically for important intellectual content: Tobias Klatte, Peter Birner, Shahrokh Shariat, Manuela Schmidinger, Martin Susani.
5. Guarantor for the article: Andrea Haitel.

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