HER2 Overexpression and Amplification in Urothelial Carcinoma of the Bladder Is Associated With *MYC* Coamplification in a Subset of Cases

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Abstract

We examined 53 invasive high-grade urothelial carcinomas (UCCs) and 42 paired lymph node metastases to determine frequency of HER2 overexpression, HER2/MYC *coamplification, and association between HER2* and *MYC status and clinicopathologic features. HER2 overexpression occurred in 19 UCCs (36%) and 14 metastases (30%), with an 88% concordance rate between UCCs and matched metastases.* HER2 *amplification occurred in 5 (10%) of 50 UCCs and 4 (11%) of 36 metastases, with a 100% concordance rate;* MYC *amplification occurred in 7 (18%) of 40 UCCs and 4 (13%) of 32 metastases, with a concordance rate of 50% between UCCs and metastases. Of 7 cases demonstrating* HER2 *amplification,* MYC *was coamplified in 4 (57%;* P = *.01), and coamplification was associated in all cases with metastasis and advanced local disease (pT4). Coamplification of* HER2 *and* MYC *occurs in a subset of patients with metastatic UCC. HER2 overexpression and amplification in metastatic lesions suggest that HER2-targeted therapy may be valuable for patients undergoing treatment for metastatic UCC, for which current therapy is limited. Further studies into the role of* MYC *coamplification in this population are needed to determine impacts on treatment with HER2-targeted therapy.*

HER2 (ERBB2) encodes a receptor tyrosine kinase of the epidermal growth factor receptor family, and increased activity of this molecule by gene amplification or protein overexpression can occur in a variety of human carcinomas.[1,2] HER2 mediates its effects via heterodimerization with other ligand-bound members of the epidermal growth factor receptor family and subsequent activation of downstream effectors such as mitogen-activated protein kinase, phosphatidylinositol-3 kinase, and MYC.[3,4] Cellular effects of HER2 signaling include cell growth, survival, and migration, and abnormal activation of HER2 has been proposed to lead to oncogenic transformation.[5] Targeting of the *HER2* gene product by the monoclonal antibody trastuzumab (Herceptin) has led to advances in the treatment of certain carcinomas, including breast carcinoma.[1,3] The impact of targeted therapy against HER2 seems to be most beneficial in the setting of gene amplification,[6] with standardized immunohistochemical analysis of the HER2 protein used as an initial screening tool to triage cases for fluorescent in situ hybridization (FISH) analysis of *HER2* gene status.

Previous studies on urothelial carcinoma have examined the expression of the HER2 protein, with a broad range of overexpression reported in 17% to 76% of noninvasive urothelial carcinomas and 23% to 80% of invasive carcinomas.[7-11] Specifically, increased protein expression of HER2 seems to correlate with earlier tumor recurrence,[10] worsened pathologic stage,[12] and decreased survival,[12-14] although the prognostic role of HER2 has been controversial and may vary depending on associated chemotherapeutic regimens.[15] To date, relatively few studies have examined the expression of HER2 in urothelial carcinoma metastases, although 1 study described frequent HER2 protein expression in this population.[8]

HER2 gene amplification has been reported in 0% to 8% of noninvasive and invasive urothelial carcinomas,[7,13,16] suggesting that protein overexpression in urothelial carcinoma is not always the result of *HER2* gene amplification. Amplification of *HER2* can occur in isolation or be associated with coamplification of other known oncogenes, including *MYC* and *TOP2A*.[17,18] Recently, studies in other cancer types have suggested that coamplification of *HER2* and *MYC* may impact tumor growth and influence response to HER2-directed therapy.[17,19] High-level *MYC* amplification (>6.0 *MYC* gene/centromere-enumeration probe [CEP]8 ratio) has been reported in approximately 3% of primary urothelial carcinomas and low-level amplification in up to 38% of cases (>2.0 *MYC* gene/CEP8 ratio in greater than 10% or 50% of cells, respectively).[20,21] However, the rates of coamplification of *HER2* and *MYC* in primary and metastatic urothelial carcinoma are unknown.

Because current chemotherapeutic regimens are frequently ineffective in patients with metastatic urothelial carcinoma,[22] identification of putative target molecules within metastatic deposits is important in the development of new therapies for patients with this disease. Although a small number of clinical trials have examined the usefulness of HER2-targeted therapy in urothelial carcinoma, the presence of *MYC* coamplification in this population, which may ultimately impact the effectiveness of HER2-targeted therapy[19] and the criteria necessary for patient selection, is unknown. We, therefore, examined a large series of invasive high-grade urothelial carcinomas and a set of paired metastases to determine the frequency of *HER2* protein overexpression and amplification and the occurrence of *MYC* coamplification in these lesions.

Materials and Methods

Tissue Specimens

Permission for this study was obtained from the Cleveland Clinic Institutional Review Board, Cleveland, OH. Paraffin-embedded radical cystectomy material obtained between the years 1999 and 2006 from 53 cases of invasive high-grade urothelial carcinoma, 44 of which demonstrated concurrent metastases at the time of cystectomy, and 42 matched pelvic lymph node metastases were available for study. All H&E-stained slides were reviewed to confirm the diagnosis, and staging was performed using the tumor-node-metastasis staging criteria from the 6th edition of American Joint Committee on Cancer staging manual.[23] Tissue microarrays (TMAs) from these specimens were constructed using three to four 1.5-mm cores from separate regions of each specimen to assess for heterogeneity of protein and gene expression. In addition, normal tissue samples included for reference and analysis in the TMAs included tonsil (3), kidney (2), liver (2), small intestine (1), placenta (1), and lung (1). Patient demographics and follow-up, obtained by a retrospective review of patient records, are summarized in **Table 1**.

HER2 Immunohistochemical Analysis

We used 3- to 4-μm sections from constructed TMAs for immunohistochemical analysis. Immunohistochemical stains for HER2 were performed according to the standardized Ventana PATHWAY HER-2/neu protocol (Ventana, Tucson, AZ). Only membrane staining was evaluated, and scoring was performed according to standard criteria[24,25] as follows: 0, no membrane staining; 1+, faint, partial membrane staining in more than 10% of cells; 2+, weak, circumferential staining in more than 10% of cancer cells; and 3+, intense circumferential membrane staining in more than 10% of cancer cells, with thick membrane staining; accompanying positive control samples were used as a reference. Protein overexpression was considered present at immunohistochemical scores of 2+ and 3+.

HER2 and *MYC* FISH

Consecutive TMAs were stained with *HER2/CEP17* and *MYC/CEP8/LPL* (Abbott Molecular/Vysis; Des Plaines, IL). Specifically, fluorescent probes used for the study included LSI *HER2* (17q11.2-q12 region) labeled with SpectrumOrange (Abbott Molecular/Vysis), LSI *MYC* (8q24.12-q24.13 region) labeled with SpectrumGreen (Abbott Molecular/Vysis), *CEP17* (alpha satellite DNA located at locus 17p11.1-q11.1) labeled with SpectrumGreen, and *CEP8* (8p11.1-q11.1) labeled with SpectrumAqua (Abbott Molecular/Vysis).

Table 1
Demographics for 53 Patients With Invasive High-Grade Urothelial Carcinoma*

Characteristic	Result
Mean (range) age, y	65 (43-84)
M/F ratio	39:14
Pathologic stage	
pT1	1 (2)
pT2	13 (25)
pT3	32 (60)
pT4	7 (13)
Patient follow-up	51 (96)
Median (range), mo	24 (1-99)
Alive with disease	18 (34)
Died of disease	33 (62)
Died of other cause	1 (2)
Lost to follow-up	1 (2)
Adjuvant therapy (n = 51)	
Chemotherapy alone	16 (31)
Radiation therapy alone	2 (4)
Chemotherapy and radiation	1 (2)
Subsequent metastases	16 (31)
Mean (range, median) time to death of disease, mo	17 (1-52, 13)

*Data are numbers (%) of cases unless otherwise specified.

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Slides were deparaffinized, and DNA target unmasking was performed using a tris(hydroxymethyl)aminomethane (Tris)–buffered saline/polysorbate (20 mmol/L Tris, 140 mmol/L sodium chloride, 0.1% polysorbate 20, pH 7.6) buffer (Cell Conditioning I/CCI prediluted solution, Ventana) for 32 minutes at 95°C. Additional peptide bond cleaving and antigen retrieval was performed using the nonspecific endopeptidase ISH Protease III (Ventana) for 8 minutes at 8°C. Slides were subsequently washed in buffer to remove unbound material, and a liquid coverslip was used throughout to minimize evaporation of the aqueous reagents from the slides. Following pretreatment, slides were removed from the staining module and washed with 2× saline sodium citrate (SSC) and water, followed by dehydration through graded ethanol washes. Slides probed with *HER2/CEP17* were denatured at 90°C for 5 minutes, and slides probed with *MYC/CEP8/LPL* were denatured at 73°C for 5 minutes using the Hybrite Chamber (Abbott Molecular/Vysis) and hybridized at 37°C overnight. Slides stained with *HER2/CEP17* were washed in 1× SSC/0.1%Np40 for 4 minutes at 72°C, and slides stained with *MYC/CEP8/LPL* were washed in 0.5× SSC/0.1%Np40 for 4 minutes at 72°C. Slides were counterstained with Vectastain Mounting Medium (4′,6-diamidino-2-phenylindole [DAPI] 2HCl; Vector Laboratories, Burlingame, CA).

Automated Scoring of FISH Results

The Metasystems Metafer Metacyte, v4.3.1.133 (Metasystems, Altlussheim, Germany), scanning system was used to scan slides. The Micro Array Tool (MAT) interface window was used to manually assign each core an ID and establish coordinate positions. The initial scans of the TMA slides were performed with the creation of a low-power overview image of the entire TMA slide, which was manually uploaded into the MAT and used to correlate the overview image with the array map containing the assigned core titles and positions. Following manual initiation, automatic FISH imaging of multiple color channels with a medium-power objective was performed in a core-by-core manner. The scanner used the coordinate position list to return to the recenter and reference object with a manual confirmation and an automatic selection of the objective. The coordinate fields were then imaged with DAPI, fluorescein isothiocyanate, SpectrumGold (Abbott Molecular/Vysis), and SpectrumAqua filter cubes individually.

Data Analysis

HER2 (17q11.2-q12) levels are reported as gene/CEP17 ratio and *MYC* (8q24.21) levels as gene/CEP8 ratio to standardize to the total chromosome number. According to previously reported criteria,[26] low-level amplification was defined as a gene/CEP ratio of 2.0 to 2.5 and high-level amplification as a gene/CEP ratio of more than 2.5. Polysomy was present when CEP8 or CEP17 levels were more than 2. Immunohistochemical and FISH results were calculated by examining 1 to 3 cores per specimen. Loss of tissue cores occurred in some cases owing to slide processing and accounts for changes in denominator designation. The average number of cores examined for each condition included 2.7 cores per primary carcinoma and 2.0 cores per paired metastatic lesion. Results from each core were averaged, with most cases demonstrating a consistent level of protein and gene copy number between cores. Association of gene amplification status was performed by using the 2-tailed Fisher exact test.

Results

HER2 Protein Overexpression Occurs in a Large Subset of Primary Urothelial Carcinomas and Associated Metastases

Examination of HER2 protein levels was performed and scored according to standardized clinical paradigms, as described. Increased HER2 protein expression (2+ or 3+) was present in 19 (36%) of 35 invasive high-grade urothelial carcinomas of the bladder **Table 2**. Of the 19 urothelial carcinomas with increased protein expression, 5 (26%) demonstrated robust (3+) levels of immunohistochemical staining. Of the 42 metastatic lesions examined, 14 (33%) demonstrated HER2 overexpression, including 7 lesions with 2+ immunostaining and 7 lesions with 3+ immunostaining **Image 1**. The concordance rate between metastatic lesion and paired primary carcinoma was 88%.

HER2 Amplification Is Present in One Third of Primary and Metastatic Urothelial Carcinomas

HER2 copy number was determined by FISH analysis using a CEP17 probe for standardization **Image 2**. Of all 53 primary carcinomas and 42 metastatic lesions examined,

Table 2
Summary of *HER2* and *MYC* Amplification and *HER2* Protein Overexpression

	Primary Carcinoma	Metastasis
HER2 protein overexpression	19/53 (36%)	14/42 (33%)
2+	14	7
3+	5	7
HER2 gene amplification	5/50 (10%)	4/36 (11%)
LLA	1	1
HLA	4	3
MYC gene amplification	7/40 (18%)	4/32 (13%)
LLA	5	1
HLA	2	3

HLA, high-level amplification; LLA, low-level amplification.

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low-level polysomy was present in 4 cases and was present in the primary tumor and the metastatic lesion in all lesions. *HER2* amplification was identified in 5 (10%) of 50 cases of primary urothelial carcinoma and consisted of 4 cases of high-level amplification (*HER2/CEP17* ratios, 3.3, 3.8, 4.3, and 8.4) and corresponding HER2 immunostains of 3+, 3+, 2+, and 3+, respectively. One case showed low-level amplification with a *HER2/CEP17* ratio of 2.0 and an accompanying 3+ immunostain.

Comparison of paired metastases with the primary lesion in the 4 cases showing *HER2* amplification identified 100% correspondence between gene amplification in the primary carcinoma and the paired metastasis, with approximately equal levels of *HER2* amplification in both lesions. Specifically, the 1 case that demonstrated low-level *HER2* amplification with a 2.0 copy number in the primary carcinoma showed corresponding low-level amplification of 2.2 in the paired lymph node metastasis. Similarly, the 3 remaining cases with high-level amplification in the primary carcinoma of 3.8, 4.3, and 8.4 copy numbers showed equivalent levels of *HER2* copy numbers in the paired lymph node metastasis of 4.0, 2.9, and 7.9, respectively. Overall, the presence of 3+ immunohistochemical staining for HER2 was more likely to predict the presence of *HER2* amplification, with 7 of 9 *HER2*-amplified

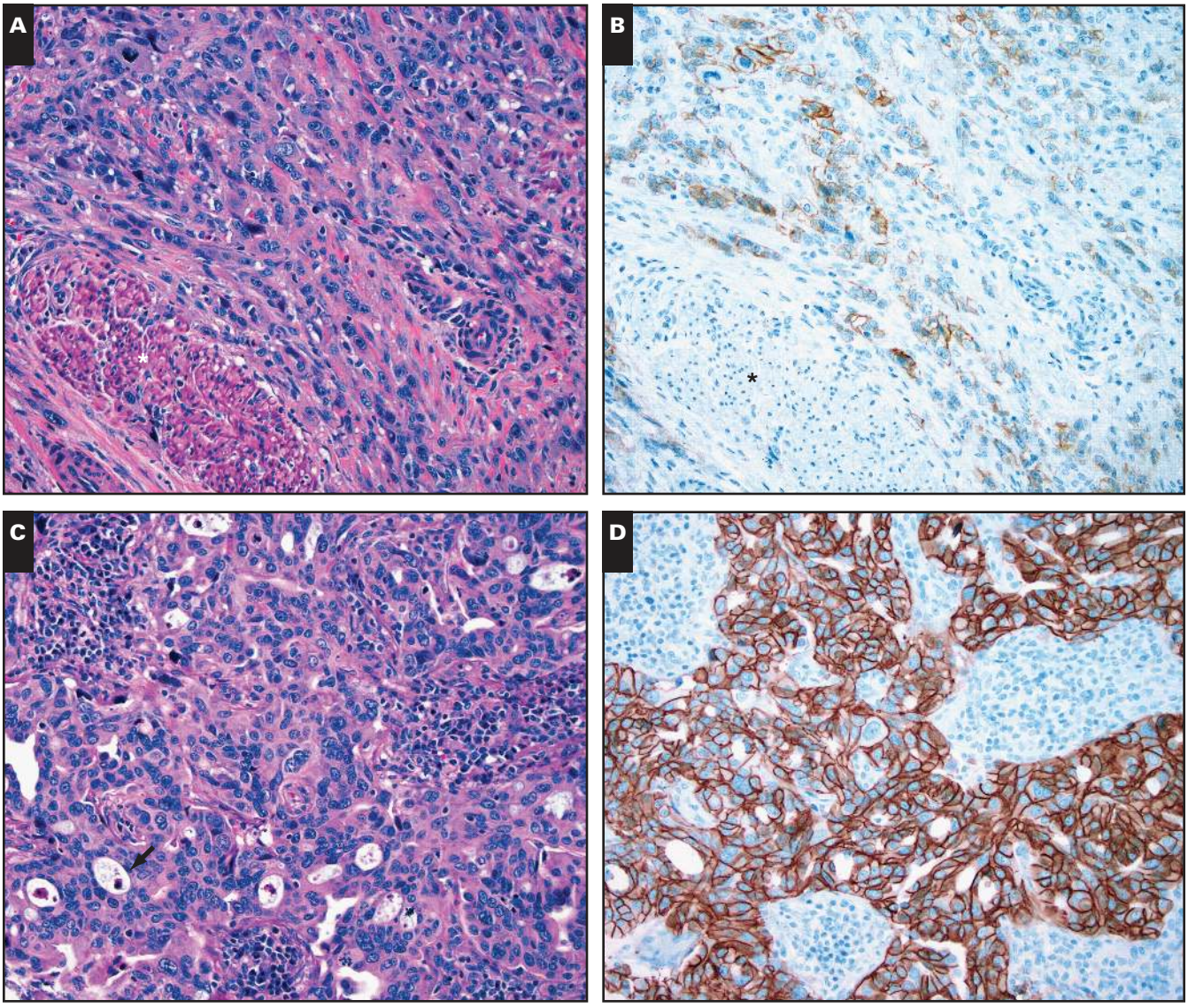

Image 1 HER2 overexpression in primary and metastatic urothelial carcinoma. **A**, Primary urothelial carcinoma involving the bladder muscularis propria (*detrusor muscle) that demonstrated concurrent metastatic disease (H&E, ×40). **B**, HER2 immunohistochemical stain of the primary carcinoma scored as 2+ (×40). **C**, Paired lymph node metastasis showing focal glandular differentiation (arrow) that was not present in the primary carcinoma (H&E, ×40). **D**, Prominent HER2 immunohistochemical stain of the metastatic lesion, scored as 3+ (×40).

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cases showing a 3+ immunostain and only 2 of 9 *HER2*-amplified cases showing a 2+ immunostain.

MYC Amplification Occurs in a Subset of Urothelial Carcinomas With Increased *HER2* Copy Number

Because *MYC* can be amplified in urothelial carcinomas[20,21] and has been shown to be coamplified with *HER2* in a subset of breast and gastric carcinomas,[17,27] we examined the association between *MYC* and *HER2* amplification in urothelial carcinoma. Of 53 primary urothelial carcinomas, 7 (13%; Table 2) demonstrated *MYC* amplification, including 5 cases with low-level amplification (2.0, 2.0, 2.1, 2.1, and 2.3) and 2 cases with high-level amplification (2.9 and 4.4). Of the 32 paired metastatic lesions available for comparison, 4 (13%) demonstrated *MYC* gene amplification with *MYC/CEP8* ratios of 2.2, 2.8, 3.1, and 5.5. Of 26 cases in which matching FISH results for *MYC* were available in the primary and metastatic lesions, the correlation between amplification in the primary carcinoma and the paired lymph node metastasis was 50% (2/4 cases). In the 2 discordant cases, *MYC* amplification was present in the primary carcinoma but not in the metastatic lesion.

Comparison of *HER2* and *MYC* amplification in primary carcinomas and lymph node metastases identified 71 total specimens in which information for both of these data points was available **Table 3**. Of 7 specimens with *HER2* amplification, 4 specimens also showed *MYC* amplification (57%) **Image 3**. The majority of the remaining cases demonstrated neither *HER2* nor *MYC* amplification. Comparison of all populations demonstrated a statistically significant association between the coamplification of *HER2* and *MYC* in urothelial carcinoma ($P = .01$).

Analysis of clinical data associated with *HER2* and *MYC* amplification, alone or in combination, identified an increased

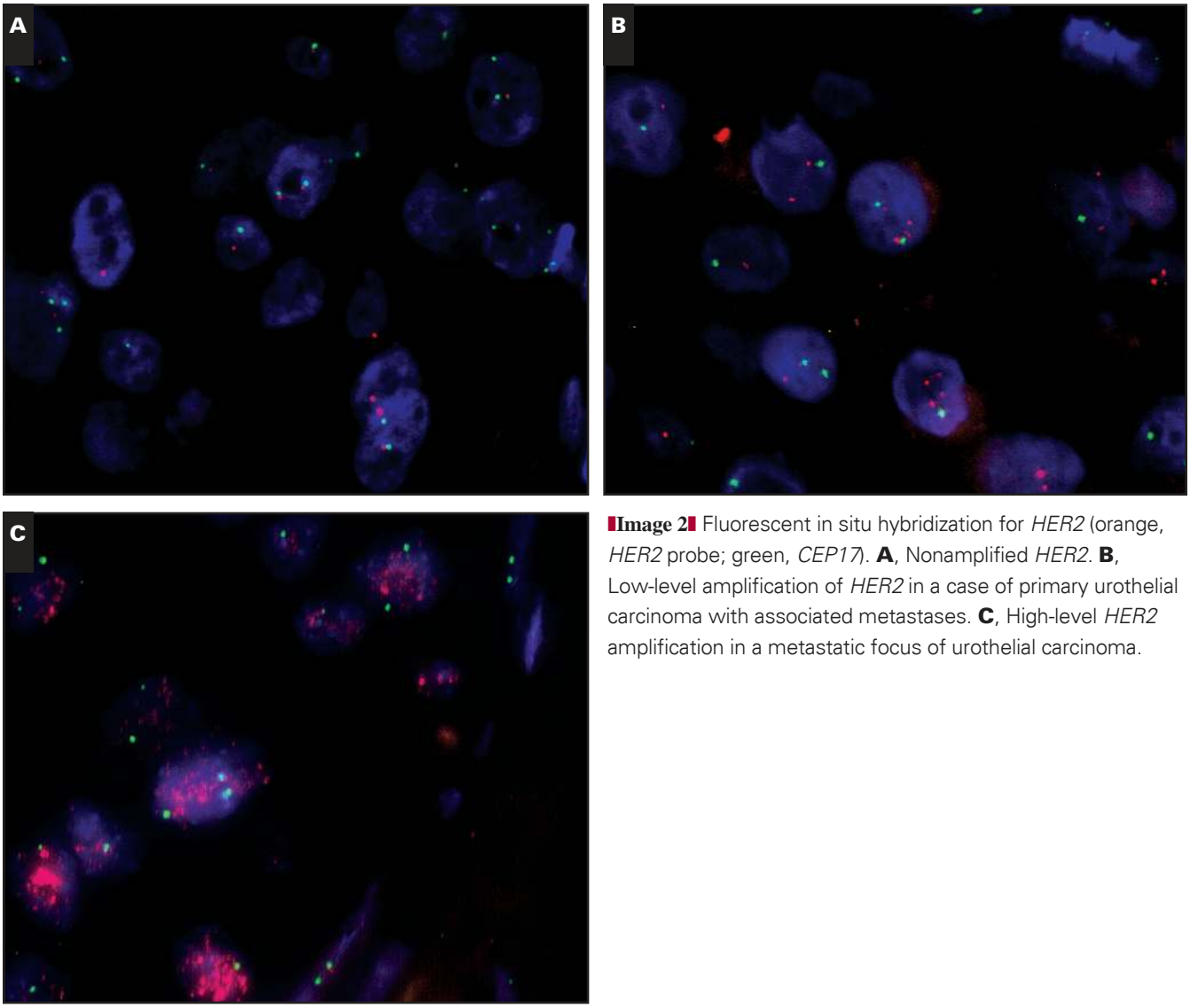

Image 2 Fluorescent in situ hybridization for *HER2* (orange, *HER2* probe; green, *CEP17*). **A**, Nonamplified *HER2*. **B**, Low-level amplification of *HER2* in a case of primary urothelial carcinoma with associated metastases. **C**, High-level *HER2* amplification in a metastatic focus of urothelial carcinoma.

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Table 3
Relationship Between *HER2* and c-*MYC* Amplification

	c-*MYC* Amplification			
***HER2* Amplification**	**Present**	**Absent**	***P***	**Total**
Present	4	3	.01	7
Absent	7	55		6

association between gene amplification and high-stage disease or worsened outcomes. Specifically, coamplification of *HER2* and *MYC* was associated in all cases (4/4) with metastatic disease and advanced local disease (pT4). All patients with *HER2* amplification died of disease (5/5; $P = .056$), whereas only 4/7 patients with *MYC* amplification died of disease, including all patients with *HER2/MYC* coamplification. No association was identified between *HER2* or *MYC* status and sex, age, or presence of subsequent metastases.

Discussion

Alterations in *HER2* by gene amplification or protein overexpression have been well-characterized in many tumor types, with *HER2* gene amplification corresponding to worsened outcomes in many studies, including those in urothelial carcinoma.[2,28] Furthermore, studies on HER2 are of particular interest in light of HER2-targeted therapies such as the monoclonal antibody trastuzumab (Herceptin).[1] Despite several studies on the overexpression and amplification of *HER2* in urothelial carcinoma, the benefits of HER2-directed therapy in urothelial carcinoma remain unclear. Because current chemotherapeutic regimens provide only a 4% to 15% long-term survival rate for patients with advanced urothelial carcinoma,[22] the identification of cellular targets within metastatic urothelial carcinoma foci is critical for the development of new treatments for this subset of patients.

In our study, HER2 protein overexpression occurred in 30% to 40% of all primary urothelial carcinomas examined, which is within the broad range of 23% to 80% reported in previous studies.[7-11] Specifically, HER2 protein overexpression seemed to be consistent between primary invasive high-grade urothelial carcinoma and metastatic deposit, suggesting that dysregulation of this pathway is maintained during the course of metastatic spread and that screening of the primary tumor may yield useful information regarding HER2 status within the metastatic lesion. Although the HER2 protein has been recently reported to translocate to the nucleus to interact with the COX-2 promoter,[29] we identified only cell membrane localization of the HER2 protein in all cases of urothelial carcinoma examined. *HER2* gene amplification occurred in approximately one third of all cases demonstrating protein overexpression, with discordant results predominantly occurring in the lesions that demonstrated 2+ rather than 3+ immunohistochemical staining. Similar to the findings identified by using protein overexpression, *HER2* amplification seemed to remain constant between the primary lesion and metastasis.

The presence of *HER2* amplification in a subset of metastatic urothelial carcinomas and the poor outcomes associated with metastatic disease in this population warrant further

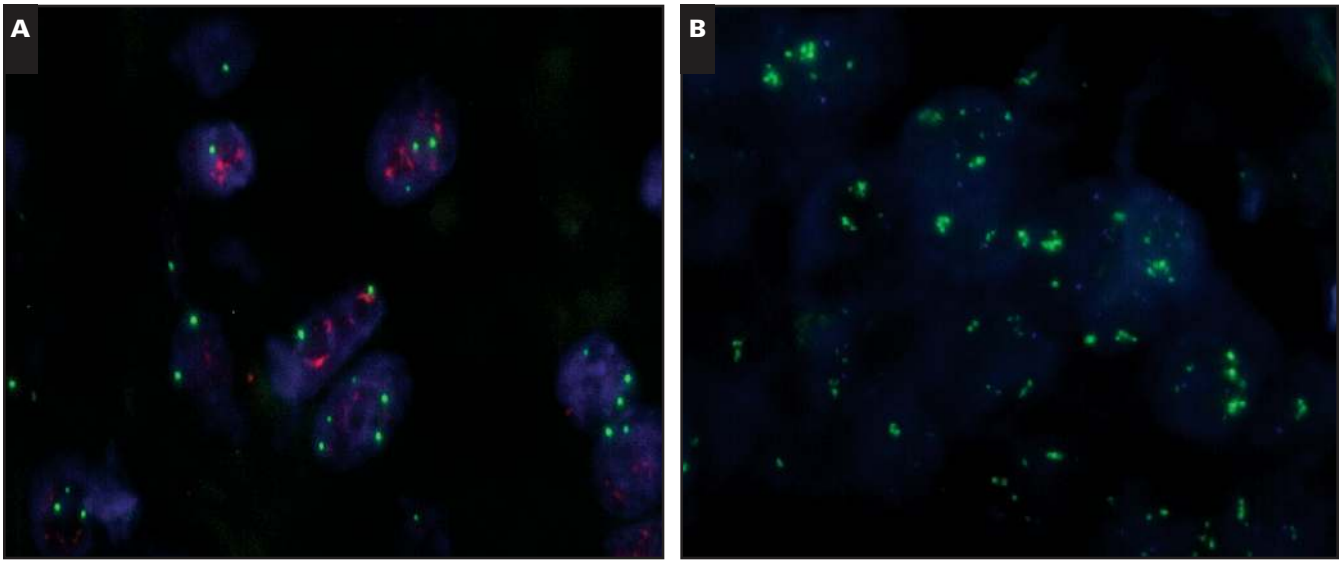

Image 3 Coamplification of *HER2* and *MYC* occurs in a subset of urothelial carcinomas. **A**, High-level *HER2* amplification (orange, *HER2*; green, *CEP17*; 4′,6-diamidino-2-phenylindole [DAPI] nuclear stain). **B**, High-level *MYC* amplification (green, *MYC*; aqua, *CEP8*; DAPI nuclear stain).

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investigation into the usefulness of targeted therapy in this population. To date, the clinical usefulness of trastuzumab treatment in urothelial carcinoma is unclear. Two groups have reported very small experiences with single-agent trastuzumab therapy in a heterogeneous group of patients with advanced urothelial cancer, with a subset of patients demonstrating some level of clinical response defined as stable disease or partial regression of metastases, although the statistical power of these studies was low.[30,31]

A larger multicenter phase 2 trial added trastuzumab to carboplatin, paclitaxel, and gemcitabine to assess the effects of this regimen on cardiac toxicity.[32] In this study, 57 (52.3%) of 109 patients were described as HER2+, although patients with 2+ HER2 immunohistochemical results were included, which may not accurately reflect *HER2* gene amplification and, therefore, subsequent response to *HER2*-targeted therapy. Despite these limitations, however, 5 of 44 HER2+ patients showed a complete response following combined treatment with complete disappearance of all disease and without the presence of new lesions for at least 4 weeks, and an additional 26 of 44 patients showed partial response following treatment, with a reduction of 50% or more in the size of measurable lesions without evidence of new or progressive disease for at least 4 weeks. Responses were present in the majority of patients who were HER2+ by using the reported criteria[32] and were highest in the group with *HER2* amplification. Further comparisons in treatment regimens with or without trastuzumab, using strict patient screening criteria, are needed to delineate the impact of combination treatment on urothelial carcinoma response.

Recently, *MYC* amplification has been reported to occur in association with amplification of the *HER2* gene in breast carcinoma and gastric adenocarcinoma (40% and 5%, respectively).[17,27] *MYC* has been proposed to function downstream of *HER2*, with coamplification impacting the proliferative status of breast cancer cells.[17] In addition, *MYC* and *HER2* coamplification has been shown to lead to worsened outcomes in patients with breast cancer following treatment with chemotherapy, which was reversed with the addition of trastuzumab to the treatment regimen.[19] Previous studies have identified *MYC* amplification in 0% to 7% of urothelial carcinomas,[20,21,33-35] although the coamplification of this oncogene with *HER2* is unknown.

We identified *MYC* amplification in 18% of primary urothelial carcinomas, which is significantly higher than reported in previous studies,[34] and identified a 50% concordance rate between primary carcinomas and metastatic lesions. Of interest, coamplification of *HER2* and *MYC* occurred only in the metastatic population in our study, and *HER2* or *MYC* amplification was associated with high-stage (pT4) disease involving adjacent organ structures. Although genetic instability may account for the finding of coamplification in any tumor type, the significant association between *HER2* and *MYC* in this study population, as well as the lack of coamplification of other oncogenes (*TOP2A* was amplified in only 1 case of invasive urothelial carcinoma; data not shown), suggests that coamplification of *MYC* with *HER2* in this population may be significant. However, the total number of cases demonstrating this finding is low and is a limitation of this study. Further investigation using a larger number of cases of metastatic urothelial carcinoma to determine the impact on clinical outcomes and effects on cellular function is warranted.

The finding in our study of the association between *HER2* and *MYC* amplification in a subset of metastatic urothelial carcinomas suggests that additional screening for *MYC* status be performed to better stratify patients and interpret clinical response to HER2-targeted therapy. Although the usefulness of HER2-targeted therapy in metastatic urothelial carcinoma is unclear, appropriate patient screening for *HER2* and *MYC* status in this population may yield important insights into patient response and may guide the development of useful therapies for patients with limited treatment options and a poor prognosis.

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