Her-2/*neu* Overexpression in Muscle-invasive Urothelial Carcinoma of the Bladder: Prognostic Significance and Comparative Analysis in Primary and Metastatic Tumors[1]

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ABSTRACT

Purpose: **The prognostic significance of Her-2/*neu* overexpression in muscle-invasive urothelial carcinoma of the bladder is largely unknown. Accurate determination of Her-2/*neu* overexpression may have therapeutic importance.**

Experimental Design: **Eighty consecutive cases of muscle-invasive urothelial carcinoma of the bladder treated by radical cystectomy with available follow-up were analyzed. In each case, one representative section was stained with anti-Her-2/*neu*. Staining was graded as 1 = faint/equivocal, 2 = moderate, and 3 = strong and was considered positive if ≥2. In those cases with a metastasis, the stain was also performed in the metastatic tumor. Results were correlated with survival.**

Results: **Twenty-two (28%) cases were considered Her-2/*neu*-positive in the primary tumor, and 17 of 32 (53%) were considered Her-2/*neu*-positive in the lymph node metastasis. Median survival for Her-2/*neu*-positive primary tumors was 33 months, compared with 50 months for Her-2/*neu*-negative cases ($P = 0.46$). Similarly, Her-2/*neu* overexpression in the lymph node metastasis did not predict survival. Sixty metastatic urothelial carcinomas were further studied by comparing Her-2/*neu* expression in the primary tumor with that of the lymph node and/or distant metastasis. Forty-five % of Her-2/*neu*-negative primary tumors had a Her-2/*neu*-positive lymph node metastasis, whereas only one case (8%) of Her-2/*neu*-positive primary tumors was Her-2/*neu*-negative in the lymph node metastasis ($P = 0.009$). Similarly, 67% of Her-2/*neu*-negative primary tumors had a Her-2/*neu*-positive distant metastasis, whereas no Her-2/*neu*-positive primary tumor was negative in the metastasis ($P = 0.429$).**

Conclusions: **Her-2/*neu* overexpression in primary or metastatic tumor did not predict survival in this cohort of muscle-invasive tumors. Overexpression in the primary tumors consistently predicts overexpression in a distant or regional metastasis. However, some Her-2/*neu*-negative primary tumors may show overexpression in their corresponding metastasis. Her-2/*neu* analysis in a metastasis may be necessary to accurately determine Her-2/*neu* status in metastatic bladder urothelial carcinoma.**

INTRODUCTION

The *H2N*[3] gene encodes a transmembrane tyrosine kinase growth factor receptor thought to control cell growth and development (1). The gene amplification and/or the protein overexpression have been associated with worse outcome in breast and ovarian carcinoma (2, 3). Recently (4), with the advent of a recombinant humanized monoclonal anti-H2N antibody (anti-$p185^{HER\text{-}2/neu}$), also known as transtuzumab or Herceptin (Genentech, San Francisco, CA), assessment of H2N status has assumed therapeutic significance. Studies (5, 6) have demonstrated that this drug, whether in combination with cisplatin or by itself, has resulted in clinical response in patients with H2N-overexpressing metastatic breast cancer.

The prognostic significance of H2N overexpression in bladder urothelial carcinoma is largely unknown, mainly because of conflicting data rendered by previous reports (7–15). Most studies have included both superficial and muscle-invasive disease in their study cohorts, so data concerning exclusively muscle-invasive disease is for the most part lacking.

Aside from its prognostic significance, accurate determination of H2N status in patients with bladder cancer may have therapeutic implications. In H2N-overexpressing breast cancer cases, there has been reported synergy between Herceptin and both paclitaxel and cisplatin (5, 16, 17), drugs commonly used in the treatment of metastatic urothelial carcinoma. Furthermore, Herceptin has been shown to have antitumoral activity when given as a single agent (6). Because H2N overexpression has been reported in up to 74% of urothelial carcinomas (18), Herceptin may have a role in metastatic bladder cancer chem-

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[3] The abbreviation used is: H2N, Her-2/*neu*.

otherapy, making accurate determination of H2N status therapeutically relevant.

We decided to perform an immunohistochemical analysis of H2N overexpression in a well-characterized set of cystectomy specimens harboring muscle-invasive urothelial carcinomas and correlate it with clinical outcome, thus evaluating its prognostic significance. Furthermore, because the pattern of H2N overexpression in metastatic *versus* primary disease is largely unknown, we performed a comparative analysis of H2N overexpression in local and/or distant metastatic disease and that of their respective primary tumor.

MATERIALS AND METHODS

Patient Selection. We collected 80 consecutive cystectomy specimens with muscle-invasive urothelial carcinomas from patients seen at Harper Hospital and the Karmanos Cancer Institute from 1990 to 1996, which had available clinical follow-up. The pathological characteristics presented in this report are reflective of the population we treat and the fact that our center is a tertiary referral center for the Metropolitan Detroit area. Under normal circumstances, radical cystectomy involved the complete resection of the bladder and prostate (in males) including complete lymph node dissection. All of the cystectomy specimens were reviewed and pathologically staged, and one representative block containing invasive tumor was selected for immunohistochemical analysis. In those cases with a regional lymph node metastasis, only one block containing metastatic tumor was also selected irrespective of the number of involved lymph nodes.

When those cases that were metastatic at presentation ($n = 32$), plus those that metastasized subsequently ($n = 17$), were grouped with an additional 11 nonconsecutive cases of metastasizing urothelial carcinomas, a total of 60 cases with regional or distant metastases were available for study. These 11 cases were added to better study the pattern of H2N overexpression in primary *versus* metastatic tumors, but because of their nonconsecutive selection, they were excluded from the survival analysis. Of these, eight cases were surgically treated with radical cystectomy, whereas in three only a transurethral resection of bladder tumor was performed demonstrating muscle-invasive disease. Four had regional lymph nodes metastasis at the time of cystectomy, and eight subsequently developed distant metastasis. In cases with distant metastasis, a block containing metastatic tumor was also selected for immunohistochemical studies.

Immunohistochemistry. Immunohistochemical staining was performed on the Ventana ES automated immunostainer. After deparaffinization, tissue sections were placed in a steamer in hot 10 mM citrate buffer (pH 6.0), processed for 20 min, and then kept in the hot buffer for an additional 20 min. Sections were then placed on the instrument and stained with polyclonal c-erbB-2 primary antibody (Dako Corp., Carpinteria, CA), diluted 1:200, for 32 min. The staining was completed with the aminoethylcarbazole dilution kit and counterstained with hematoxylin.

Her-2/*neu* Overexpression Scoring. Stained sections were reviewed independently by two pathologists. In all of the cases, the pathologists were blinded to the clinical outcome. Scoring was done using the following system: 1+, those tumors showing at most faint, equivocal, and incomplete membranous staining (Fig. 1*A*); 2+, unequivocal, complete membranous pattern, with moderate intensity (Fig. 1*B*); and 3+, those tumors that showed areas of strong, membranous pattern (Fig. 1*C*). Tumors with scores of 2 or 3 were considered as H2N-positive.

Metastatic tumors in lymph nodes and distant sites were graded using the same scale, without knowledge of the staining pattern of the corresponding primary tumor (Fig. 1, *D* and *E*).

We also assessed the degree of overexpression heterogeneity within each H2N-positive tumor. For the purposes of this study, we arbitrarily defined heterogeneous overexpression as the presence of at least one low power field of completely absent staining in an otherwise H2N-positive tumor (Fig. 1*F*).

Statistical Analysis. For the first set of 80 cases, histological features and H2N overexpression were correlated with survival. The median follow-up period for the study was 44.3 months. Survival analysis was done using the Kaplan-Meier and log rank test.

For all of the cases, frequencies of data were analyzed using Fisher's exact test. Statistical significance was reached if a $P < 0.05$ was obtained.

RESULTS

Survival Analysis. For the initial population of 80 patients, the mean age was 62.9 years, with a male:female ratio of 2.2. All of the tumors were high-grade muscle-invasive urothelial carcinomas treated by cystectomy.

Table 1 shows the distribution of cases by T stage and lymph node status. Twenty-six (33%), 32 (40%), and 22 (27%) cases were pT2, pT3, and pT4, respectively. Thirty-two cases (40%) had positive metastasis to regional lymph nodes at time of cystectomy, whereas lymph nodes were negative in 32 patients (40%). In 16 cases (20%), the lymph node status was not pathologically assessed at time of cystectomy.

Fifty-eight (72%) primary tumors were considered H2N-negative (staining intensity of 0 or 1+ in 41 and 17 patients, respectively), and 22 (28%) were considered H2N-positive (staining intensity of 2+ or 3+ in 8 and 14 patients, respectively). The median survival of H2N-negative patients was 50 months and that for H2N-positive patients was 33 months ($P = 0.46$). No significant difference was found in T-stage distribution or incidence of lymph node metastasis between H2N-positive and H2N-negative tumors (Table 2). When H2N overexpression was assessed in regional lymph node metastases, 17 (53%) of metastatic tumors were H2N-positive (staining intensity of 2+ or 3+ in 7 and 10 cases, respectively), and 15 (47%) were H2N-negative (staining intensity of 0 or 1+ in 14 and 1 cases, respectively). The median survival for patients with H2N-negative *versus* H2N-positive lymph nodes were 24 and 28 months, respectively ($P = 0.39$).

The median survival for the entire group was 43.6 months. There was a significant difference in survival among the different T stages, as well as between those cases with and without lymph node metastasis (Fig. 2, *A* and *B*). However, H2N overexpression did not predict survival when analyzed by its presence either in the primary tumor or in the regional lymph node metastasis (Fig. 3, *A* and *B*). We repeated our analysis using only 3+ staining as criterion for H2N-positive status. By this

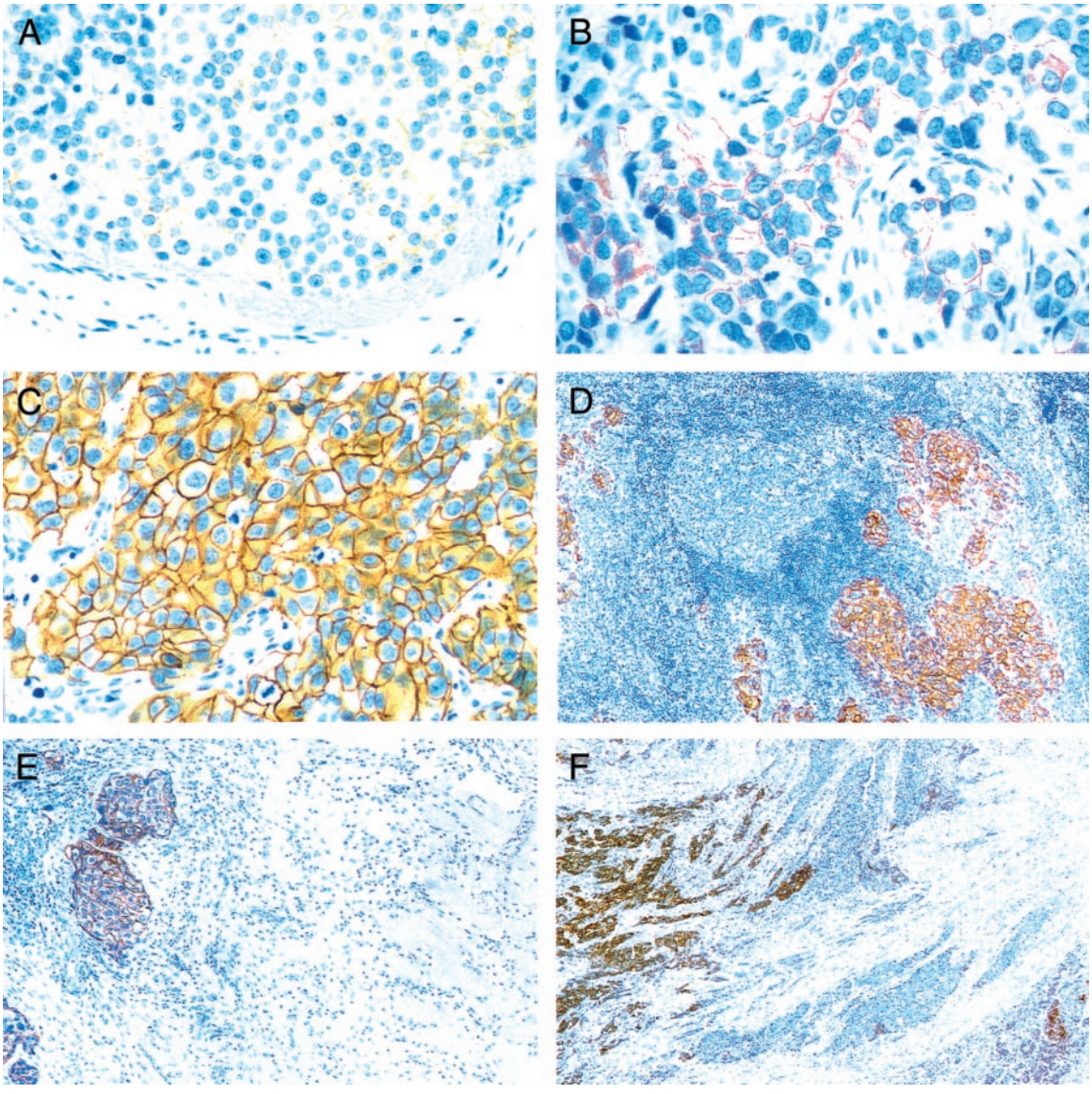

Fig. 1 Grading of H2N overexpression by immunohistochemistry. *A*, 1+; *B*, 2+; and *C*, 3+. Tumors with 2+ or 3+ were considered H2N positive. H2N overexpression in metastatic tumors was graded using the same scale, without knowledge of the overexpression pattern of the primary tumor. *D*, H2N overexpression in metastatic tumor to a regional lymph node; *E*, H2N overexpression in metastatic tumor to a distant site. In this case, nests of metastatic carcinoma are seen infiltrating amid skeletal muscle fibers of the chest wall. *F*, H2N overexpression heterogeneity. For the purposes of this study, overexpression heterogeneity was defined as the presence of at least one low power field of completely absent overexpression in an otherwise H2N-positive tumor.

criterion, 14 of 80 (18%) primary tumors and 10 of 32 (31%) metastatic tumors in lymph nodes were H2N-positive. Similarly, no correlation with survival was noted when only 3+ tumors were considered.

Overexpression in Primary *versus* Metastatic Tumors. Table 3 shows the distribution of these cases by T stage and lymph node status at the time of surgical treatment of the 60 cases of metastatic urothelial carcinoma. Nineteen cases had only lymph node metastasis, 24 had only distant metastasis, and 17 had metastasis to both regional lymph nodes and distant sites. All 60 (100%) of primary tumors, 32 of 36 (89%) of tumors with metastasis in regional lymph nodes, and 7 of 41 (17%) of distant

Table 1 Distribution of consecutive cystectomy cases by T stage and lymph node status with associated median survival

T stage	LN Neg[a]	LN Pos	Unknown LN status	Total	MS (m)
T2	14	10	2	26	69
T3	14	11	7	32	50
T4	4	11	7	22	16
Total	32	32	16	80	
MS (m)	66	32		41.4	

[a] LN, lymph node; Neg, negative; Pos, positive; MS, median survival; m, months.

Table 2 Distribution by T stage and incidence of lymph node metastasis in Her-2/*neu*-positive and -negative primary tumors from consecutive cystectomy cases

H2N status	T2	T3	T4	LN (+)[a]
H2N-negative	33%	41%	26%	50%
H2N-positive	32%	36%	32%	50%

[a] LN (+), lymph nodes positive for metastatic carcinoma.

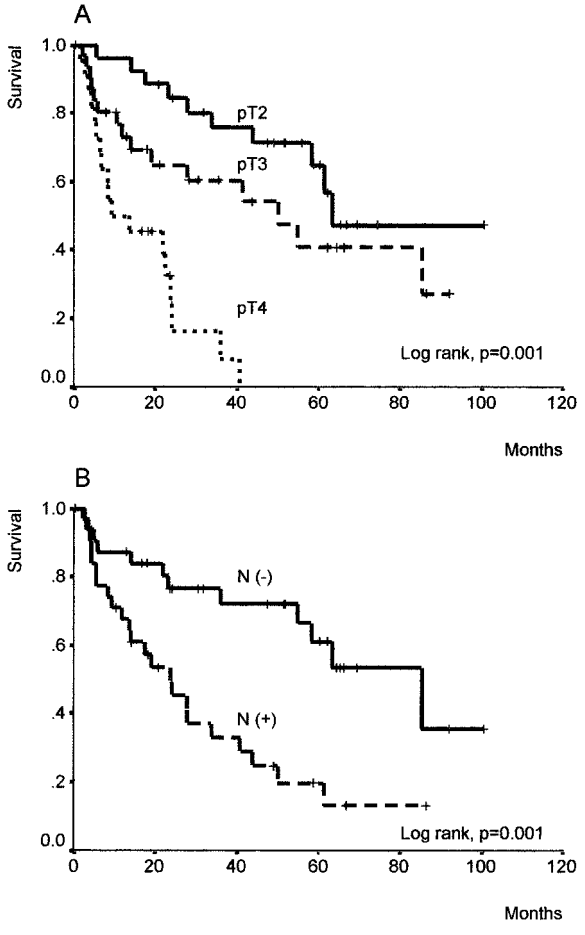

Fig. 2 Survival in consecutive cystectomy specimens according to T stages (*A*) and lymph node status (*B*).

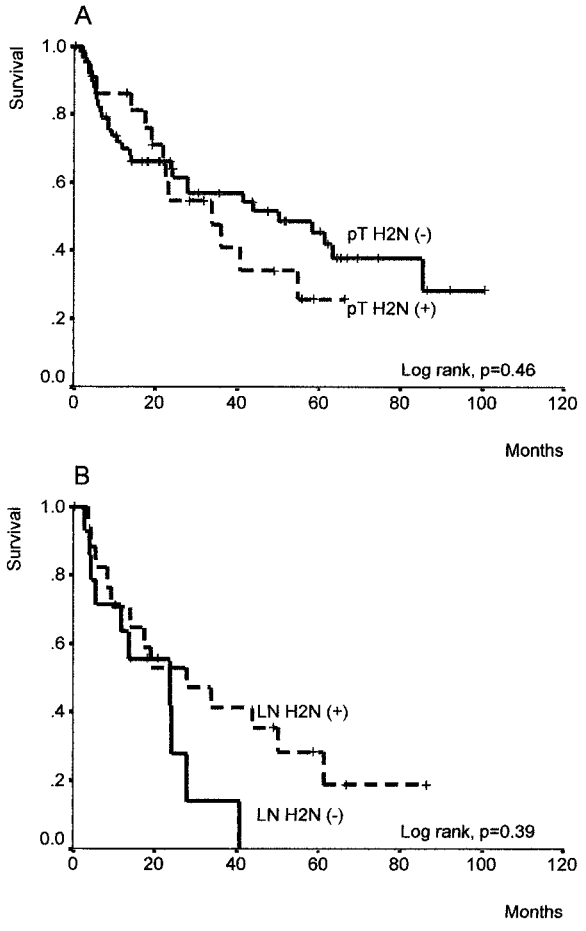

Fig. 3 Survival according to Her-2/*neu* overexpression (*A*) in the primary tumor (*PT*) and (*B*) in the lymph node (*LN*) metastasis.

metastatic tumors had available tissue for immunohistochemical studies. Three cases have available tissue from both a regional lymph node and a distant metastasis.

H2N overexpression was seen in 37% (22 of 60) of primary tumors, 63% (20 of 32) of regional lymph nodes metastases, and 86% (6 of 7) of distant metastases ($P = 0.008$). Tumor overexpression heterogeneity, as defined above, was present in 64% of primary tumors in the section examined.

Of the 32 cases with positive lymph node metastasis, 45% of H2N-negative primary tumors were associated with a H2N-positive lymph node metastasis, whereas only one case (8%) of H2N-positive primary tumors was H2N-negative in the lymph node metastasis ($P = 0.009$). Similarly, for the seven tumors that had available tissue from a distant metastasis, 67% of H2N-negative primary tumors were associated with a H2N-positive distant metastasis, whereas no H2N-positive primary tumor was negative in the distant metastasis ($P = 0.429$; Table 4). Three cases had tissue from all of the three sites (*i.e.,* the primary tumor, a regional lymph node metastasis, and a distant metastasis). In one case only, the distant metastasis was H2N-positive. In the second, both metastases were positive and the primary tumor was negative, and in the third, the primary tumor and distant metastasis were H2N-positive, with the regional lymph node negative.

DISCUSSION

The significance of prognostic factors in urothelial carcinoma is highly dependent on the stage of the disease and the standard treatment for that particular stage. Management of noninvasive or superficially invasive (*i.e.,* pTa/pT1) bladder tumors is focused on local control of the disease. Useful prognostic factors in this setting are those that can predict recurrences or progression to muscle invasion. On the contrary, presence of muscle-invasive disease (*i.e.,* pT2, pT3, or pT4

Table 3 Distribution of metastatic cases by T stage and lymph node status at time of surgical treatment and subsequent development of distant metastasis

T Stage	Unknown LN[a] status	LN Neg	LN Pos	Total	
				n	%
T2[b]	4	5	12	21	35%
T3	4	5	16	25	42%
T4	3	3	8	14	23%
Total	11 (18%)	13 (22%)	36 (60%)	60	100%
No. of cases that developed a subsequent distant metastasis	11	13	17	41	68%

[a] LN, lymph node; Neg, negative; Pos, positive.
[b] Three cases had transurethral resection as the only surgical treatment.

Table 4 Comparison of Her-2/*neu* overexpression in metastatic tumors to lymph nodes and distant sites with their corresponding primary tumor

Her-2/*neu* in primary tumor	Her-2/*neu* in LN metastasis[a]			Her-2/*neu* in distant metastasis		
	Neg	Pos	Total	Neg	Pos	Total
Negative	11 (55%)	9 (45%)	20	1 (33%)	2 (67%)	3
Positive	1 (8%)	11 (92%)	12	0 (0%)	4 (100%)	4
Total	12	20	32	1	6	7
P	0.009			0.429		

[a] LN, lymph nodes; Neg, negative; Pos, positive.

tumors) has traditionally been considered indicative for aggressive surgical therapy (*i.e.,* radical cystectomy), which includes assessment of regional lymph nodes to rule out metastasis. In turn, presence and/or development of local or distant metastases are usually indications for systemic chemotherapy in bladder cancer. Thus, relevant prognostic factors in nonmetastatic muscle-invasive disease are those that can predict development of local or distant recurrences. In the setting of metastatic disease, however, predictors of survival, quality of life, and response to systemic therapy are the most useful indicators.

The *H2N* gene is localized on chromosome 17q and encodes a M_r 185,000 transmembrane tyrosinase kinase growth factor receptor (1, 19). Although its ligand has not yet been fully identified, activation of the intracellular kinase is thought to play a role in cell differentiation, motility, and adhesion (20). Overexpression and amplification of the oncogene was first identified in a human breast carcinoma cell line (21) and, subsequently, in approximately 30% of breast adenocarcinoma (3). It has been repeatedly shown to be a prognostic marker in breast cancer, particularly in lymph node-positive patients (2, 22, 23). Its prognostic significance in other epithelial neoplasms, however, is less clear.

Reports of H2N expression in urothelial carcinoma have ranged from 2 to 74% (7–15, 24). Moreover, its prognostic significance is highly controversial, with several conflicting results in the published literature. Thus, although some studies (12, 14) have correlated H2N overexpression with a more aggressive clinical behavior, others (7, 9, 13) have found no prognostic significance association, and others (8, 10, 11, 15) have linked it to a better clinical outcome. Differences in both the incidence of expression and its prognostic significance are very likely explained by different methodologies, including assessment of H2N status (*i.e.,* detection of amplification *versus* detection of overexpression), method used (*i.e.,* PCR, fluorescence *in situ* hybridization, and immunohistochemistry), and definition of H2N positivity. Thus, the literature in the field is extremely difficult to compare, and significant conclusions are hard to draw from the aggregate data. Our study assessed H2N overexpression by immunohistochemistry, which is the method most commonly used for breast cancer H2N-status determination, and with a widely used antibody, which is the basis for the commercially available Hercept Test. Controversies regarding the optimal way of assessing H2N status (*i.e.,* gene amplification *versus* overexpression) are current in breast cancer literature (25–27). When compared with breast cancer, overexpression without gene amplification appears to be significantly more common in bladder cancer, where it has been reported in up to 36% of cases (28). In the series of Zhau *et al.* (18), 70% of tumors showed evidence of H2N protein overexpression by Western blot, whereas only 8% of them had evidence of gene amplification by Southern blot. Others (9, 13, 29–31) have found similar results. Although at this point the clinical significance of overexpression without amplification is uncertain, the majority of the studies evaluating H2N as a predictive factor for response to either conventional chemotherapy or Herceptin have been based on immunohistochemical data (32). Notwithstanding, the limitations of immunohistochemistry cannot be overemphasized and include dependency on quality of tissue fixation, cross-reactivity of antibodies with other epitopes, and interobserver variations in scoring and interpretation of immunostains. Our study failed to identify a significant difference in survival in tumors with and without H2N overexpression. Furthermore, because of the ongoing controversy in the breast cancer literature on whether 2+ tumors should be considered H2N-positive (25, 26, 33), we repeated our analysis using only 3+ staining as criterion for H2N-positive status. By this criterion, 14 of 80 (18%) primary tumors and 10 of 32 (31%) metastatic tumors in lymph nodes were H2N-positive. Similarly,

no significant difference in survival was seen in H2N-positive and H2N-negative tumors (data not shown).

Recently, the main interest in H2N has been focused on its therapeutic predictive value. With the advent of the humanized monoclonal anti-H2N antibody transtuzumab or Herceptin, accurate determination of H2N status has acquired therapeutic implications (28). In preclinical studies (34), muMab 4D5, the murine version of Herceptin, produced significant antiproliferative effects *in vitro* against human breast cell lines that overexpressed the H2N receptor, whereas it had no effect on cell lines that did not overexpress it. Herceptin binds the extracellular domain of the H2N receptor with three times greater affinity than does muMab 4D5 and induces an antibody-dependent cellular cytotoxicity against tumor cell lines (35). The clinical benefit of Herceptin has been demonstrated in a recent Phase III trial, which has shown a more prolonged time to disease progression in patients treated with Herceptin and chemotherapy, compared to those treated with chemotherapy alone, in women with metastatic breast cancer with H2N overexpression (16). Similarly, Herceptin has been associated with a 15% response rate in patients with metastatic H2N-positive breast cancer when given as a single agent (6). On the other hand, Herceptin has been associated with a syndrome of myocardial dysfunction similar to that observed with anthracyclines (6). Thus, it is apparent that accurate determination of H2N status is of high therapeutic significance, because patients who would benefit most from Herceptin can be accurately identified, minimizing unnecessary risks to those that will not benefit from the drug.

There is no data on the efficacy of Herceptin in the treatment of metastatic bladder cancer. However, synergy between paclitaxel and Herceptin in breast cancer has been reported (16, 36), and recent studies (5, 17) have suggested a potential synergy between Herceptin and cisplatin. These two drugs are part of the armamentarium of the medical management of metastatic bladder cancer, and thus it is at least possible that Herceptin may have a role in the management of metastatic bladder cancer, especially considering the significant proportion of urothelial tumors that overexpress H2N. A Phase II clinical trial is currently in progress in our institution, evaluating the efficacy, toxicity, and feasibility of a combination of Herceptin, paclitaxel, carboplatin, and gemcitabine in the treatment of patients with urothelial metastatic carcinoma.

Our study demonstrated that determination of H2N status in urothelial carcinoma is dependent on whether the assessment is done in primary or metastatic tumor. Incidence of H2N overexpression was significantly higher in metastatic tumors compared with primaries. Furthermore, we identified a fairly reproducible pattern of overexpression when metastatic tumors were compared with their corresponding primary. H2N-positive primary tumors consistently developed H2N-positive metastasis; however, H2N-negative tumors were associated with H2N-positive lymph node metastasis in 45% of cases and H2N-positive distant metastasis in 67% of cases. Although this difference could be explained arguably by the acquisition of H2N-positive phenotype as the tumor develops metastatic potential, our finding of frequent heterogeneity in the overexpression pattern of urothelial carcinoma suggests another possibility. It could be theorized that an H2N-positive component in the primary tumor was not represented in the block chosen for immunohistochemical analysis and that this component was the one that (perhaps preferentially) developed metastatic disease and, thus, was more likely to be present in the sampling of the metastasis. Both scenarios would imply a significant relationship between H2N overexpression and metastasis development, which, however, did not translate into prognostically significant data in our survival analysis. Unfortunately, we had only three cases with available tissue from the three tumor sites, precluding further analysis of the relationship between H2N and metastatic progression. In any event and most importantly, it appears that H2N-status determination is more accurate when performed in a metastatic tumor than in a primary tumor, especially if the primary tumor is negative. Others (8, 28) have found evidence of H2N-overexpression heterogeneity in bladder cancer. Discordance in H2N overexpression between metastatic and primary tumor in prostate cancer has also been reported recently (37). In breast cancer, however, concordance of H2N status between primary and metastatic appears to be higher (38–40), probably because of the fact that overexpression heterogeneity is seen less frequently.

The main limitation in our study was tissue availability and the use of only one representative sample from lymph node metastases. There was a significant difference in tissue availability depending on the site of the tumor. We had a significantly low percentage of distant metastatic tumors that were sampled and that rendered sufficient tissue to perform immunohistochemical analysis. This is secondary to the fact that distant metastasis, in a patient with a known primary tumor, is rarely sampled, and if done, it is usually through a fine needle aspiration, which usually does not render enough quantity of tumor to perform immunohistochemical analysis. Thus, our data suggest that if Herceptin proves to have therapeutic value in the management of metastatic urothelial carcinoma, a more aggressive pursuit of tissue sampling from distant metastasis may be indicated to accurately determine H2N status, especially in patients with H2N-negative primary tumors.

In summary, we have identified H2N overexpression in 28% of primary urothelial carcinomas and found no prognostic significance with regard to survival. Overexpression analysis in primary *versus* metastatic tumors was discordant in a significant proportion of cases, especially in the presence of a H2N-negative primary tumor. H2N-status determination appears to be more accurate when performed in metastatic tumor, especially when the primary tumor is negative. This preliminary finding, if corroborated, may have clinical implications at the time of selecting patients for Herceptin therapy in urothelial carcinoma.

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