Journal of BUON **14:** 457-462, 2009

ORIGINAL ARTICLE

Prognostic impact of HER2/neu protein in urothelial bladder cancer. Survival analysis of 80 cases and an overview of almost 20 years' research

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Summary

Purpose: *This study was conducted to evaluate the quantitative assessment of HER2/neu immunohistochemical expression in urothelial bladder cancer in order to determine its prognostic significance.*

Materials and methods: *Archival tumor tissue from 80 patients with primary urothelial carcinoma were analysed for HER2/neu immunohistochemical expression. A highly reproducible standardized procedure on a Bond-X automated slide stainer was used.*

Results: *HER2 protein was overexpressed in 41 of 80 patients (51.25%), demonstrating an increase in the expression rate corresponding to progressively advanced tumor stage (p=0.032) and tumor grade (p=0.0001). Kaplan-Meier analyses showed that positive membranous expression of HER2/neu was not associated with an increased probability of tumor recurrence (p=0.362). In contrast, HER2 scores correlated strongly with specific survival probability (p=0.002) and overall survival (p=0.025). Multivariate analysis revealed that only stage was an independent predictor of specific survival (p=0.016). HER2 expression was an independent predictor of specific survival with borderline statistical significance (p=0.08).*

Conclusion: *HER2 overexpression represents a prognostic factor for adverse disease outcome.*

Key words: HER2/neu, prognosis, urothelial carcinoma

Introduction

HER2 is a transmembrane glycoprotein involved in cell growth control. It represents a significant target for immunological based antitumor therapy owing to its limited expression in nonmalignant tissues and its contribution to the malignant phenotype of a transformed cell. This protein has been extensively investigated in urothelial bladder cancer but its clinical relevance as prognostic variable remains unclear. Varying rates and conflicting data persist in the literature regarding its association to bladder cancer outcome. The reported overexpression ranges from 8.2% to 81% [1,2]. These discrepancies reflect the heterogeneity of these studies and can be attributed to different factors, such as: absence of quality controls in the preanalytic and analytic steps of HER2 overexpression assay; different methods of evaluation (definition of positive expression, validated scoring system); the use of different monoclonal antibodies and cutoff values. Moreover, technical disparities concerning the degree of trypsinization, incomplete blocking of endogenous peroxidase activity, temperature and pH may all influence the intensity of reaction obtained with a particular chromophore system. Advancements in quality standards of immunohistochemical assays necessitate a re-examination of some significant biomarkers that once had presented contradictory conclusions in various studies.

The objective of our study was to investigate the potential prognostic impact of HER2 protein utilizing

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Received 13-05-2009; Accepted 09-06-2009

a highly reproducible standardized procedure on a Bond-X automated slide stainer (Vision biosystems bond, Newcastle, UK).

Materials and methods

Study population

The material comprised 80 specimens of urothelial carcinoma of the urinary bladder obtained by transurethral resection or total cystectomy at our institution from April 1998 to May 2005. The cohort of patients in this study was highly selected, fairly homogeneous and treated in a prospective and standardized fashion. Samples were processed anonymously. The demographic information, clinical presentation, pathologic stage, and follow-up were extracted from the medical charts. Tumor grading and staging were determined according to the principles outlined by the World Health Organization (WHO 2004) and TNM classification of the International Union Against Cancer (UICC). The study population consisted of 69 men and 11 women with a mean age of 65 years (range 26-85) and a mean follow up time after initial diagnosis of 33.9 months (range 12-96).

Immunohistochemistry

One representative paraffin block was selected for each case. The major criterion of selection was the good presentation of morphology. Cauterized or quantitatively inadequate material was avoided. Only sections containing sufficient epithelium to assess the antibody reactivity on 1000 cells were considered eligible for this study. A mouse monoclonal antibody (clone CB11, Novocastra, Newcastle, UK) at 1:40 dilution was used. Immunohistochemistry (HIC) protocol for this antigen was carried out on 4 μm thick paraffin sections of the corresponding blocks. The marker was applied to the sections using a Bond-X automated staining system (Vision biosystems bond, Newcastle, UK). This specific assay is based on a soluble dextran-polymer system which yields a high signal to noise ratio and minimizes any background that may be caused by endogenous biotin. Polymer Based Detection technology is an advancement in immunohistochemical visualization chemistry. After peroxidase blocking the sections were incubated with primary antibody for 60 min at room temperature and then incubated with horseradish peroxidase labeled polymer - HRP LP for 30 min. The antigen-antibody reaction was visualized using 3-3′diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate. Finally, tissue sections were slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. The omission of the primary antibody in simultaneously incubated sections was used as negative control. A known positive breast carcinoma was included as positive control. 300 cells were examined and selected among at least 5 non consecutive fields and chosen in the most viable areas of the lesions at × 400 magnification in order to quantify HER2/neu expression. Only membranous staining was considered as positive, being consistent with c-erbB2 protein's role as a transmembrane molecule. The c-erbB2 expression level was classified into 4 groups according to published guidelines [3]. These scores are defined by a lack of staining or membranous staining in less than 10% of the cells (score 0), faint or barely perceptible membranous staining in more than 10% of the cells (score 1), complete membranous staining in more than 10% of the cells of weak to moderate intensity (score 2) and complete membranous staining in more than 10% of the cells of strong intensity (score 3). In this scoring system, scores of 2 or higher are considered to indicate HER2 overexpression. All immunohistochemical slides were analyzed by two independent pathologists. The interobserver variability was low. Discordant observation were resolved by attainting consensus. Assessment of all staining results was blinded to knowledge of the clinical outcome of patients.

Statistical analysis

Correlation of HER2/neu expression with clinical parameters was calculated using the x^2 test. Disease-free survival and overall survival rates were estimated using the Kaplan-Meier algorithm for incomplete observations. Cumulative survival curves were compared using the log-rank test. The overall survival time was defined as the interval between the date of diagnosis and the last date when the patient was known to be alive (censored) or date of death for any reason (uncensored). The disease-free survival rate was measured as the period of time between the date of diagnosis and the date of the last follow-up examination in which the patient was disease-free (censored), or the date of first recurrence independently if this was local, regional, or distant recurrence (uncensored). The disease specific survival rate was defined as the time period between date of diagnosis and time of death. Patients who died from causes other than urothelial carcinoma were not counted in this estimation. A Cox proportional hazards ratio model was used to determine independent predictors of relapse-free and overall survival using factors

significant on univariate analysis as covariates. In all cases, a p value ≤0.05 was considered to be statistically significant. Statistical analysis was performed using the SSPS software program 16.0 (SSPS Inc, Chicago, IL, USA).

Results

Associations between HER2 expression and clinicopathologic factors are tabulated in Table 1. A summary of the results of the x^2 analysis of the studied variables is also displayed in the same Table. According to the adopted scoring system, we found a cell membrane overexpression (score 2,3) in 51.25% (41/80) of the cases. Expression patterns of HER2/neu staining are depicted in Figure 1. There was an obvious predominant cell membrane staining pattern in 51 cases (score 1,2,3). A faint cytoplasmic staining was detected in all cases, probably due to the reaction of antibody against various epitopes of cytoplasmic domain of HER2.

During the follow up time, disease recurred in 29 of 66 patients with superficial tumor (44%) and progressed in 15 patients (23%). When writing this paper, 31 patients had died. Of these, 25 died of metastatic bladder cancer and 6 of other causes without evidence of disease progression. In statistical analysis HER2/neu expression was taken as a dichotomous variable: negative (score 0, 1+) vs. positive (2+, 3+). A strong relation between membranous HER2 positive staining and increased grade (p=0.0001) and stage (p=0.032), was proved, as determined by the x^2 test. Kaplan-Meier analysis showed that positive membranous expression of HER2/neu was not associated with an increased probability of tumor recurrence (p=0.362; Figure 2). On the contrary, the HER2 scores correlated strongly with overall survival (p=0.025; Figure 3), and disease specific survival probability (p=0.002; Figure 4) analyzing all patients.

Using the Cox proportional hazards methods, we performed a multivariate analysis to assess the independent predictive value of all significant markers for

Table 1. Correlation between clinicopathological parameters and HER2/neu staining pattern

Parameters	*Cases*	*Cell membrane expression*		
		0,1+	*2+,3+*	*p-value*
Gender				
Male	69	32	37	0.614
Female	11	6	5	
Age (years)				
<65	30	18	12	0.083
≥65	50	20	30	
Grade				
Low	52	33	19	0.0001
High	28	5	23	
Stage				
Superficial	66	35	31	0.032
Muscle invasive	14	3	11	

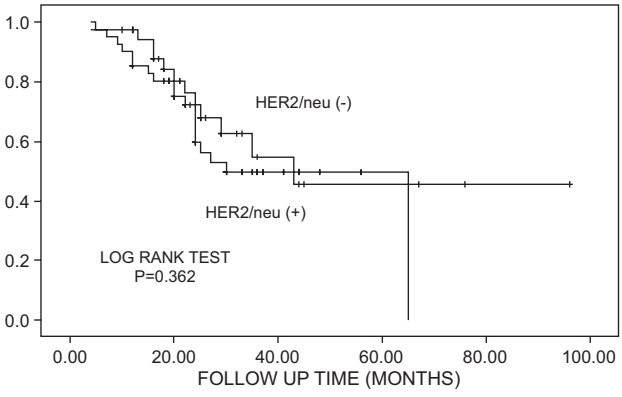

Figure 2. Relapse-free survival by cell membrane expression of HER2.

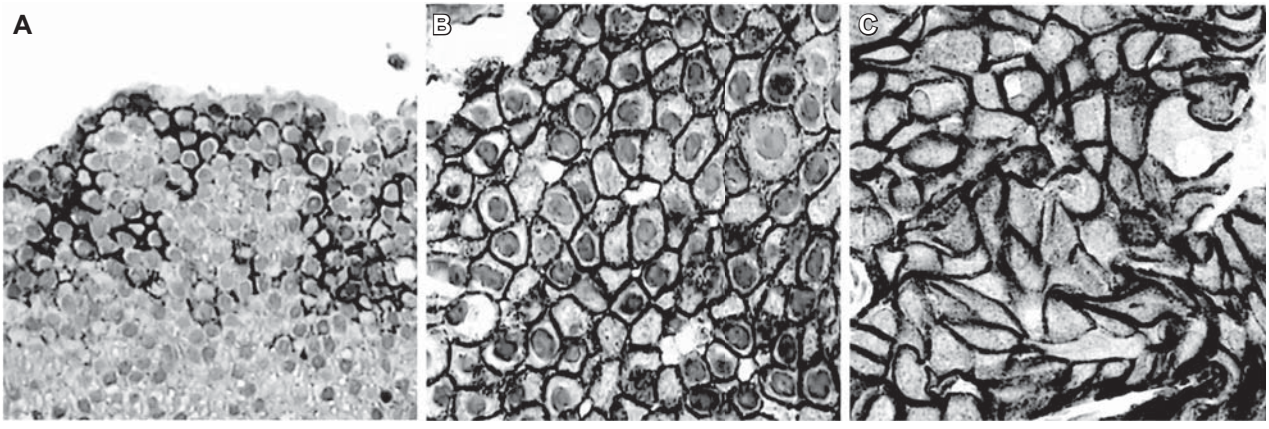

Figure 1. Immunohistochemical demonstration of HER2 protein. Staining scores are displayed. **A:** Score 1+, **B:** Score 2+, **C:** Score 3+ (×200, ×400, ×400, respectively).

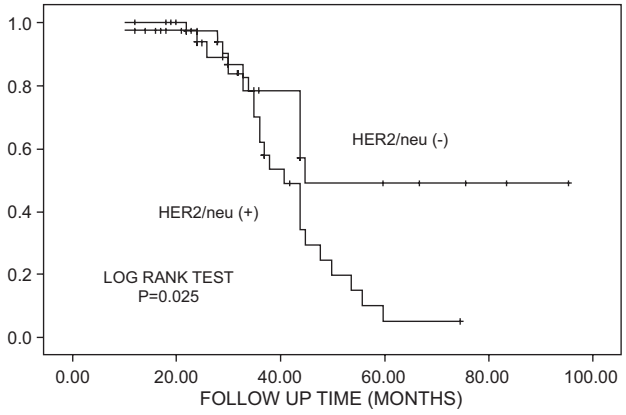

Figure 3. Overall survival by cell membrane expression of HER2.

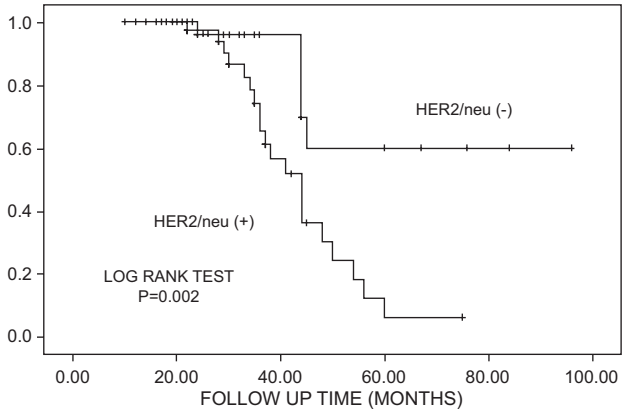

Figure 4. Univariate analysis of disease specific survival by cell membrane expression of HER2.

the disease specific survival (Table 2). The following prognostic variables were included in the model: HER2/neu membrane expression (positive, negative), stage (superficial, muscle invasive) and grade (low, high). The multivariate analysis revealed that only stage was an independent predictor of disease specific survival (p=0.016; Table 2). The HER2 expression was an independent predictor of disease specific survival with borderline statistical significance (p=0.08).

Table 2. Cox proportional hazard model analysis

	Bladder cancer specific survival		
	Hazard ratio	*p-value*	*95%CI*
Grade	2.596	0.344	0.513- 6.805
Stage	3.671	0.016	1.277-10.557
HER2/neu expression	1.868	0.08	0.118- 1.127

Grade: low vs. high, stage: superficial (PTa, PT1) vs. muscle invasive tumors (≥PT2), HER2/neu membrane expression: positive (score 2+, 3+) vs. negative (0,1+), CI: confidence interval

Discussion

Urothelial carcinoma is the second most common cancer of the genitourinary tract [4] and has the highest recurrence rate of any cancer. It presents a significant heterogeneity in biological behavior that is not completely understood. It is widely known that two morphologically similar tumors presenting in any assigned stage may behave in different fashions, a fact that seriously impedes the potentiality to accurately predict clinical outcome in a given case. The imperative clinical need for a prognostic indicator in urothelial bladder carcinoma is well established. To date none of the candidate prognostic markers add some predictive ability beyond that offered by the conventional clinical and pathological parameters.

HER2 encodes an 185kD orphan receptor tyrosine kinase that is constitutively active as a dimer and displays potent oncogenic activity when overexpressed [5]. There is mounting evidence of the role of HER2 overexpression in patients with breast carcinoma. It has been amply demonstrated that HER2 overexpression is associated with intensive proliferation and aggressive development of breast cancer [6]. Immunohistochemically detected HER2 has been solidly correlated to poor prognosis in 26-30% of breast tumors [7]. HER2 overexpression occurs to a spectrum of several other human cancers, including carcinomas of the ovary [8], uterus [9], lung [10], salivary glands [11], stomach [12] and colon [13]. Most of these studies have documented an association of HER2 overexpression with inferior prognosis.

Although there is a large number of anti-HER2 antibodies (each targeting different epitopes on the HER2 receptor), the most commonly used are the polyclonal antibody A0485, used alone or as part of the HercepTest, and the monoclonal antibodies CB11 (alone or as part as the Ventana PathWay kit) and TAB250. Several investigators have reported that the sensitivity and specificity of anti-HER2 monoclonal and polyclonal antibodies differ, although a number of these studies have also shown a high rate of concordance among the different anti-HER2 antibodies. It is important to be aware of the specificity and sensitivity of individual antibodies and to take into account that antigen retrieval increases the sensitivity of the antibody at the expense of specificity. It is generally accepted that monoclonal antibodies are more specific than polyclonal antibodies and the use of the latter may result in a higher false-positive rate.

HER2/neu encodes a membrane receptor and its product is localized mainly onto the cytoplasmic membrane. There are several putative explanations for the

cytoplasmic localization of staining, including suggestions that it represents an intermediate or alternative protein product [14-16]. The biologic significance of this cytoplasmic staining is not clear.

Many previous studies have investigated the prognostic implications of HER2 in urothelial bladder cancer patients, with inconsistent and contradicting results. Most studies have included both superficial and muscle-invasive disease in their study cohorts. A few studies have included exclusively muscle-invasive diseases.

Studies including advanced stage of disease (muscle-invasive)

Kroger et al. [17] reported one of the strongest associations in a study group of 203 patients with muscle-invasive carcinoma. HER2 demonstrated an overexpression in 37% of the cases and this was associated with grade and infiltrative growth pattern. On multivariate analysis, HER2 overexpression was an independent predictor of disease specific survival. Another study found no correlation between HER2 staining and survival in 80 muscle-invasive cystectomy specimens [18]. The bladder tumor stained for HER2 in 28% of the cases and lymph node metastases, when present, stained for HER2 in 53% of the cases. Interestingly, in patients with nodal or distant metastases, 45% and 67% of those without HER2 in the primary tumor showed HER2 staining in the lymph node or distant metastasis, respectively. Loss of HER2 staining in a metastasis compared with the primary tumor was rare. It would therefore appear to be critical to assess HER2 status in metastases in order to determine tumor HER2 status most accurately. HER2 positive tumors were associated with more metastatic sites in a cohort of patients with advanced disease captured prospectively as part of a clinical trial investigating the role of HER2-targeted therapy [19].

Studies including disease of all stages

It has been clearly documented in 4 studies [20-23] of this category that expression of HER2 correlates strongly with tumor grade and stage. HER2 protein has showed an increase in the expression rate corresponding to the advancement of tumor grade and stage. These results are in line with our findings. Sato et al. [20] could verify its independent importance in predicting patients' survival. They reported that the HER2 expression, tumor grade, tumor stage were independent variables with respect to patient prognosis and that HER2 immunohistochemical status was the most significant prognostic factor after tumor stage. Principally, in our study tumor stage was an independent factor of prognosis and secondarily HER2 expression was an independent factor with borderline significance. These data together with our findings suggest a prognostic relevance of HER2 protein overexpression. In one series of 179 patients with all stages of bladder cancer, HER2 overexpression was observed more frequently in pT1 tumors (74%) than in pTa (49%) or pT2-T4 (56%) tumors [24]. Metastases were found in 66% of patients with HER2 staining in the primary tumor, but in only 37% of those lacking HER2 staining. Two studies have specifically analysed T1 patients, reporting that the recurrence rate was greater in T1 tumors expressing HER2 compared with T1 tumors lacking HER2 [25].

Patients with advanced disease have a poor prognosis after radical cystectomy, even with adjuvant chemotherapy [26]. The low survival rates of these patients, even with standard chemotherapy, emphasize the need for other therapeutic strategies. The majority of these patients have a high rate of HER2/neu overexpression. With the development of targeted anti-HER2 therapies, assessment of HER2 status will be important in stratifying patients to the most appropriate treatment regimens. Hence, it can be speculated that trastuzumab might become a therapeutic option in advanced bladder cancer. Some preliminary data suggest that trastuzumab-based therapy may be safe and effective in metastatic transitional cell carcinoma of the urinary tract [27].

The prognostic and predictive significance of HER-2/neu protein still constitutes one of the thorniest fields in urothelial bladder cancer research. However, inconsistencies between studies do exist and this is likely to be due in part to different testing methods used to determine HER2 status. Our data corroborate the findings of other studies that HER2 overexpression is high in patients with advanced bladder cancer and influences the outcome of these patients, thereby suggesting that HER2 is a potential target for the treatment of this cancer.

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