



# The Survey of Her2/neu Expression and It's Correlation with Some Pathological Factors in Urothelial Bladder Tumors

Samane Danaei,<sup>1</sup> Seyed Hamid Madani,<sup>1,\*</sup> Sedigheh Khazaei,<sup>1</sup> Babak Izadi,<sup>1</sup> Elahe Saleh,<sup>2</sup> and Soraya Sajadimajd<sup>3</sup>

<sup>1</sup>Molecular Pathology Research Center, Imam Reza University Hospital, Kermanshah University of Medical Sciences, Kermanshah, Iran

<sup>2</sup>Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

<sup>3</sup>Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

\*Corresponding author: Dr Seyed Hamid Madani, Molecular Pathology Research Center, Emam Reza Hospital, Zakaria Razi Bol, Kermanshah, Iran. Tel: +98-8334283392, Fax: +98-8334282906, E-mail: shmmadani@yahoo.com

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## Abstract

**Background:** Overexpression of human epidermal growth factor receptor 2 (Her2) protein has been shown in a variety of human malignant tumors. In some of these tumors, such as breast carcinoma, targeted anti-Her2 therapy has been considered as a promising therapeutic strategy.

**Objectives:** The aim of this analytic descriptive study was to evaluate the immunohistochemical expression of Her2 and its relation to grade and muscular invasion of urothelial bladder tumor.

**Methods:** In this descriptive-analytic study, 179 paraffin-embedded tissue blocks from patients with urothelial bladder tumors were selected for immunohistochemistry (IHC) analysis using antibodies against Her2. IHC staining was evaluated according to the same criteria of breast carcinoma.

**Results:** Her2 overexpression was detected in 49 patients (27.4%) and was significantly associated with high tumor grade and bladder muscular involvement ( $P < 0.001$ ).

**Conclusions:** Her2/neu over expression is associated with aggressive behavior of urothelial bladder tumors. Assessment of Her2 status could be helpful to identify patients with a high probability of disease progression. Anti-Her2 therapy could be used in urothelial bladder cancers, however, this issue needs further studies.

**Keywords:** Bladder Tumor, HER2-neu, Immunohistochemistry

## 1. Background

Bladder carcinoma is the second common malignancy of urogenital system and the sixth common malignancy in the United States. Bladder malignancy encompasses 5.2% of all forms of cancer and is the fifth common malignancy in Iran with a 4-fold prevalence in men than in women. The median age was about 56 years old (1, 2). Transitional cell carcinoma (TCC) accounts about 92% of bladder malignancies (3). Smoking, exposure to arylamines, aniline dyes, auramines, phenacetin cyclophosphamide, and Schistosoma hematobium infections are considered as the known risk factors involved in urothelial carcinoma (4).

Urothelial bladder tumors are grossly divided into papillary and nonpapillary (flat) lesions. Papillary neoplasms are structurally and cytologically classified into papilloma, papillary urothelial neoplasm of low malignant potential, low-grade papillary urothelial carcinoma, and high-grade

papillary urothelial carcinoma based on 2004 WHO classification. According to involvement of muscularis propria of the bladder, it categorized as muscle invasive and noninvasive groups. Urothelial bladder carcinomas can spread into the neck of the bladder, urethra, prostate ducts, and seminal vesicles. The most common sites of distal metastasis are the lungs, liver, bone, and central nervous system (5).

Her2/neu is a transmembrane protein with tyrosine kinase activity that belongs to epidermal growth factor receptor family (6). Overexpression of Her2 is assessed by IHC and FISH assays with a good correlation. In most cases, IHC is the first method to evaluate the expression of Her2 that is reported as 0, 1+, 2+, and 3+. Detection of Her2/neu overexpression is an efficient prognostic technique for response to Herceptin. Frozen and fixed tissues in formalin and also cytological slides that fixed in alcohol are used for study by IHC and/or FISH methods (7). Overexpression and amplifi-

cation of Her2 has been reported in urothelial bladder cancer (8). Immunostaining of Her2 was shown in cytoplasm and membrane of cells in urothelial tumors (9, 10). The expression of Her2 has been suggested to increase with the stage and recurrence of urothelial cancer (11-13). According to a meta-analysis study, Her2 expression is correlated with poor prognosis and can be considered as a prognostic clinical biomarker for bladder cancer (14).

All things considered, the aim of this study was to evaluate the expression of Her2 in 179 patients with urothelial bladder tumor and its association with some pathological parameters of this tumor.

## 2. Methods

### 2.1. Materials

c-erbB-2 oncoprotein A\*0485, Biotin Blocking System, Liquid DAB + Substrate Chromogen System, EnVision and Dual Link System, and Target Retrieval Solution were provided from DAKO (Denmark). *Hematoxylin* was purchased from Panreac (Spain). Hydrogen peroxide, Methyl alcohol, Entelan glue, and Ethyl alcohol 99.6% were supplied from Merck (Germany). Pepsin was provided from Sigma (Germany). Xylene and Ethyl alcohol 96% - 70% were purchased from shimi-lab (Iran). All used experimental apparatus were standard and calibrated.

### 2.2. Patient Samples

In this analytic descriptive study, 179 urothelial tumor specimens including four radical cystectomy tissues and 175 biopsies provided by transurethral resection from 2012 to 2014 were analyzed at Imam Reza Educational Hospital, Kermanshah, Iran. The slides prepared from paraffin blocks of the specimens were stained with the conventional hematoxylin and eosin method. All specimens were reviewed and original diagnoses were confirmed by two independent pathologists. The sample size was determined by accident and calculated using the following formula:

$$n = \frac{Z_{1-\frac{\alpha}{2}}^2 P(1-P)}{d^2} \quad (1)$$

In which, n = Sample size;  $Z_{1-\alpha/2}$  = Confidence interval; P = estimated proportion and d = desired precision.

Therefore,

$$n = \frac{1.96^2 0.35(1-0.35)}{0.07^2} \approx 179 \quad (2)$$

The inclusion criteria for samples include tissue samples with at least 70% tumoral tissue in surface area and

adequate level of muscaris propria to evaluate the involvement of muscle by tumor. The exclusion criteria were based on samples that lost their qualities after preparation and immunohistochemistry. In addition, admission committee was received from Kermanshah University of Medical Sciences by a registration code number of KUMS.REC.1394.47 at 2014.

### 2.3. Hematoxylin and Eosin (H and E) Staining

Paraffin-embedded tissues were provided as 4  $\mu$ m sections and stained with hematoxylin and eosin (H and E) method. In this method, glass slides containing tissue sections incubated at 70°C for 2 hours. Then, slides rinsed into several jars filled with xylene, graded series of ethanol solutions, hematoxylin, lithium carbonate, and eosin. Stained sections were independently evaluated by two pathologists.

### 2.4. Immunohistochemistry

Immunohistological staining was performed on formalin-fixed paraffin embedded tissue sections using antibodies against Her2. For this aim, 4  $\mu$ m tissue sections were deparaffinized at 37°C for 2 hours and Xylene for 24 hours. Then, slides rehydrated in a graded series of ethanol solutions and PBS (phosphate buffered saline) for about 12 minutes. To retrieve antigens, slides immersed in the jar containing Tris buffer (pH = 9) and heated in a water bath at 95°C for 20 minutes followed by washing in PBS solution. To quench the intracellular activity of peroxidases, slides were immersed in a solution of 3% Hydrogen peroxide in methanol for 10 minutes, washed with PBS and placed in jars containing avidine solution for 5 minutes. Then, biotin was added to increase the specificity of staining. After washing with PBS, slides were incubated by primary and secondary antibodies for 45°C and 30°C, respectively in a humid and dark place at room temperature. The slides were washed in PBS and stained with substrate - chromogen solution known as 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 minutes. The counterstaining was performed with hematoxylin for 30 seconds and washed in water. The stained slides immersed in graded series of ethanol and then, xylene to transparency and dehydration of tissues. Then, slides mounted to the study under a microscope. Negative controls were exposed to antibody diluent replacing primary antibody. Her2 positivity was assessed according to recommendations of the American society of clinical oncology/college of American pathologists 2013 Her2 test guideline established for breast cancer (15). Her2/neu has a membrane staining that is scored based on complete or incomplete staining and the intensity of staining of the

tumor cells. Score 0 presents no membrane staining or less than 10% of the tumor cells; score 1+ presents faint membrane incomplete staining in more than 10% of the tumor cells; score 2+ presents a weak or moderate complete membrane staining in more than 10% of the tumor cells; score 3+ presents a strong complete membrane staining in more than 30% of the tumor cells.

### 2.5. Statistical Analysis

Data were analyzed using the SPSS (V.18). The correlation between expression of Her2 and tumor grade and other variables were assessed with t-test and chi-square tests. All differences were considered statistically significant at the level of  $P < 0.05$ .

## 3. Results

In this study, 179 cases of bladder urothelial tumors were evaluated including 4 (2.2%) radical cystectomy specimens and 175 (98.8%) transurethral resection specimens. The median age of patients is 67.74 with the range of 14 to 92 years old. The frequency of patients based on sex, age, grade of the tumor, and involvement of muscle were summarized in [Table 1](#).

**Table 1.** The Frequency of Patients Based on Age, Gender, Muscular Involvement and Tumoral Grade

Characteristics	Number	Percentage
<b>Age, y</b>		
≤ 60	69	38.5
> 60	110	61.5
<b>Gender</b>		
Female	26	14.5
Male	153	85.5
<b>Muscle involvement</b>		
Present	65	36.3
Absent	114	63.7
<b>Tumoral grade</b>		
PNLMP	59	33
Low grade carcinoma	73	40.8
High grade carcinoma	47	26.2

Strong protein expression (3+) was observed in 49 of 179 tumors (27.4%). An immunohistochemistry 0/1+ scores were detected in respectively, 33 (18.4%) and 57 (31.8%). A total of 40 cases (22.3%) were scored as 2+ ([Figure 1](#) and [Table 2](#)). There was no a meaningful relation between the age and gender of patients with the expression of Her2/neu ( $P$  value = 0.148 and 0.33, respectively). Significant difference was

observed between various grades of urothelial bladder tumors at the level of Her2 expression ( $P$ -value  $< 0.001$ ) as tumor grade progresses, expression of Her2 is increasing.

**Table 2.** The Frequency of Patients with Different Her2 Scoring

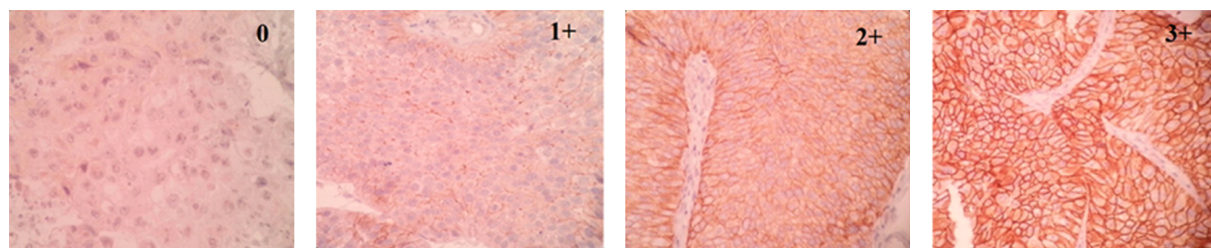
HER 2/neu Scoring	Number	Percentage
0	33	18.4
1	57	31.8
2	40	22.3
3	49	27.4
<b>Total</b>	179	100

Also, there was a significant difference between the expression of Her2/neu and the status of muscular involvement ( $P$ -value  $< 0.001$ ). Overexpression of Her2/neu was observed in 29 cases (44.6%) of tumors with muscular invasion, whereas in 20 cases (17.5%) of tumors without muscular involvement, strong expression was detected. More details about the frequency of patients with different Her2 scoring based on varieties were listed in [Table 3](#).

## 4. Discussion

In this study, 179 urothelial bladder tumors, including 153 men (85.5%) and 26 women (14.5%) with an average age of 64.74 years old was evaluated to determine the expression and association of Her2/neu with some pathological parameters of urothelial tumors. The expression of Her2/neu was negative (score 0 and 1+) in 90 cases (50.3%), suspicious (score 2+) in 40 cases (22.3%), and positive (score 3+) in 49 cases (27.4%).

Diagnosis of biomarkers such as Her2/neu oncoprotein can be applied for target therapy using novel and/or recombinant chemodrugs such as trastuzumab, which is examined in Her2 positive cancers including breast cancers. Wide variations were observed at the level of Her2 expression in several previous studies. Due to the variability in kits, methods, solutions, and subjective interpretation of staining result, this difference could happen. A research entitled with "Immunohistochemical Study of Her2/neu Expression in Urothelial Bladder Carcinoma", in Egypt in 2015, has reported that among 60 paraffin embedded tissues of urothelial bladder tumors, 29 patients (48.3%), 9 cases had score 2+ and 20 cases were score 3+. In addition, there was a meaningful statistical relation between Her2/neu expression and tumor grade ( $P$ -value  $< 0.001$ ). However, there was not a meaningful relation between Her2/neu expression and tumor stage ( $P$ -value = 0.16). Furthermore, the association between Her2/neu with gender, tumor size, and lymph node involvement were not shown

**Figure 1.** Immunohistochemical Evaluation of Her2 in Urothelial Bladder Tumors

IHC staining of Her2/neu (scores of 0, 1+, 2+ and 3+) by magnification of 400 × in urothelial bladder tumors.

**Table 3.** The Frequency of Patients with Different Her2 Scoring Based on Varieties<sup>a</sup>

Variables	0	1	2	3	Total, n = 179
<b>Age, y</b>					
≤ 60	13 (18.8)	28 (40.6)	12 (17.4)	16 (23.2)	69 (100)
> 60	20 (18.2)	29 (26.4)	28 (25.4)	33 (30)	110 (100)
<b>Gender</b>					
Female	4 (15.4)	11 (42.3)	7 (26.9)	4 (15.4)	26 (100)
Male	29 (18.9)	46 (30.1)	33 (21.6)	45 (29.4)	153 (100)
<b>Tumoral grade</b>					
PNLMP <sup>b</sup>	6 (10.2)	32 (54.2)	15 (25.4)	6 (10.2)	59 (100)
Low grade carcinoma	19 (26)	18 (24.7)	16 (21.9)	20 (27.4)	73 (100)
High grade carcinoma	8 (17)	7 (14.9)	9 (19.2)	23 (48.9)	47 (100)
<b>Muscular invasion</b>					
Present	12 (18.5)	12 (18.5)	12 (18.5)	29 (44.6)	65 (100)
Absent	21 (18.4)	45 (39.5)	28 (24.6)	20 (17.5)	114 (100)

<sup>a</sup>Data are presented as N.(%).

<sup>b</sup>Papillary neoplasm of low malignant potential.

(16). Among 160 patients who were evaluated by Nedjadi et al., the expression of Her2 (+2, +3) was about 60% using IHC. Further, there was a strong relationship between Her2 expression with lymph node involvement and tumor grade (P value < 0.05) (17). In another study by Behzatoglu et al., from 60 patients with micropapillary urothelial carcinoma, 34 cases (56%) showed an overexpression of Her2 (3+). In addition, they proposed that the expression of Her2 in micropapillary urothelial carcinoma was stronger than other types of this carcinoma (18).

Slim Charfi and colleagues have studied the expression of Her2/neu in 151 paraffin embedded urothelial bladder carcinoma tissues. They reported that the overexpression of Her2 (score 3+), p53 and low expression of p62 using immunohistochemistry was positive in 14 cases (9.3%), 48 cases (31.8%), and 58 cases (38.4%), respectively. There was a meaningful association of the expression levels of

Her2, p53 and low level of p62 with higher grades (P-value = 0.0002) and also the TNM stage in advanced tumors. Besides, multivariate analysis has been shown that there was only a meaningful relation between tumor grade and Her2 expression (P-value = 0.006) (19).

Skagias I and coworkers in the study related to the prognostic effect of Her2/neu protein in bladder urothelial cancer used archival paraffin tissues from 80 patients with urothelial bladder cancers. They have been shown that the expression of Her2/neu protein using immunohistochemical staining was positive in 41 of the 80 patients (51.25%) that had a meaningful relationship with stage (P = 0.032) and grade of the tumor (P = 0.0001). In fact, the expression of Her2/neu was an independent prognostic factor of survival with a borderline statistical significance (P = 0.08). Authors have been suggested that the expression of Her2 is a valuable prognostic factor to diagnose the adverse con-

sequences of disease in patients (20). Stefan Kruger et al., have studied the expression of Her2/neu in urothelial cancer with invasion to bladder muscle in Germany. In 57 cases (41%) of 138 patients with invasive intramuscular urothelial bladder carcinoma, Her2/neu was overexpressed that was increased with advanced grades of the tumor ( $P = 0.03$ ). there was no meaningful statistical relationship between Her2, tumor staging, and lymph node involvement. The results have been shown that Her2 could be used as a prognostic factor in patients with urothelial cancer (21).

As compared to previous studies, we tried to use the large number of samples to best evaluate the interplay of Her2 expression and characteristics of patients. In addition, the involvement of bladder wall muscle as a variable and determination of its association with Her2 expression in urothelial bladder tumors was conducted in this study. It has been suggested that involvement of bladder muscle is one of the poor prognostic factors in survival of patients with bladder cancer. However, the accuracy of the results was influenced by personal and laboratory interference, subjective interpretation of IHC results, retrospective status of study, and the lack of patients' accessibility.

#### 4.1. Conclusion

Among 179 urothelial bladder tumors, overexpression of Her2/neu (score 3+) was observed in 49 cases (27.4%). Her2/neu overexpression was significantly correlated with tumoral grade and bladder muscular involvement ( $P < 0.001$ ). However, results showed no meaningful relationship with the expression of Her2/neu and patient's age ( $P = 0.148$ ) and gender ( $P = 0.33$ ).

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