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ORIGINAL ARTICLE

The Effect of C-erbB-2 Expression on Clinical-Pathological Prognostic Factors and Survival Ratios in Urothelial Tumors

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Abstract

Introduction: Overexpression and the amplification of proto-oncogenic c-erythroblastic oncogene B (c-erbB-2) (HER-2/neu) encode transmembrane growth factor glycoprotein and are a part of the type I receptor tyrosine-protein kinase. It is known to play a role in the treatment of breast and stomach carcinomas. C-erbB-2 overexpression and amplification in urothelial carcinomas have also been demonstrated in many studies. However, possible treatment options and prognostic values are still not sufficiently defined. This study aims to determine the overexpression rate of c-erbB-2 in urothelial carcinomas. In addition, we sought to determine if there was a relationship between c-erbB-2 overexpression and clinical-pathological prognostic factors, such as histological grade, pathological stage, lymph node and distant organ metastasis, and survival time.

Methods: C-erbB2 overexpression was examined using an immunohistochemical method performed on paraffin block samples of patients diagnosed with primary bladder urothelial carcinoma (n=41). The relationship of the data obtained after this examination with classical clinical-pathological prognostic factors, such as histological grade, pathological stage, regional lymph node metastasis, distant organ metastasis, and survival, was investigated. The obtained data were evaluated statistically. The alpha significance value was 0.05.

Results: C-erbB-2 overexpression was positive in 9.75% (4/41) of the cases. c-erbB-2-positive tumors were all found to be at a high level. One patient was in stage T2, one stage T3, and two in stage T4. Three positive cases had lymph node metastasis; one patient did not have lymph node metastasis. Remote organ metastasis was not observed in any positive cases, and all positive cases were reported to survive. The fact that 75% (3/4) of the positive cases were lymph node metastasis may contribute to the literature.

Discussion and Conclusion: Standardized laboratory methods should be used and studied in large series, including more patients representing various races to evaluate c-erbB-2 overexpression and amplification status in urothelial tumors as a treatment option with prognostic value.

Keywords: C-erythroblastic oncogene B-2 overexpression; survival; urothelial carcinoma.

Bladder cancers rank seventh in men and seventeenth in women worldwide[1]. Approximately 90% of bladder malignant tumors are urothelial carcinomas[1]. From 75% to 85% were superficial (Ta, Tis, and T1), and 15-25% were invasive (T2-T4) or in the metastatic stage when urothelial bladder carcinomas were first diagnosed[2]. More than 70% of superficial bladder tumors recur after the first treatment, with approximately one-third progressing to an advanced histological grade[2]. The cure rate is between 20% and 50%, even though radical surgery, radiotherapy, and

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chemotherapy are coadministered in invasive tumors[2].

The frequent recurrence and progression of tumors with a low histological grade increases the importance of knowing the markers of progression in these tumors when the first diagnosis is made. Clinical-pathological parameters such as histological grade, pathological stage, and lymph node metastasis in bladder tumors are conventional prognostic factors used to predict recurrence, progression, and survival in patients with bladder urothelial tumors. However, these traditional prognostic factors do not accurately predict the progression of the disease in some cases[3]. Therefore, other prognostic markers are needed to accurately assess the progression of urothelial tumors, response to treatment, and long-term survival rates. Accordingly, scientists focused on protein and molecular markers while conducting their research on urothelial tumors[4].

Protein kinases, which play a role in the carcinogenesis of bladder tumors, are mediators that provide cell proliferation, migration, adhesion, and transformation[3,5].

Erythroblastic oncogene B (erbB) receptor family members constitute a large group of the epidermal growth factor receptor, erbB-1, erbB-2, erbB-3, and erbB-4 receptor tyrosine-protein kinase family.[3,5]. A study in the literature reported overexpression or amplification of the erbB receptor family as a molecular marker in bladder tumors and many other tumors[6]. The human monoclonal antibodies trastuzumab (Herceptin®)[7] and pertuzumab (Perjeta®), which have inhibitory capabilities against c-erbB-2, are used in breast and stomach tumors that are HER2 receptor-positive or represent c-erbB-2 overexpression and amplification. The frequency and prognostic significance of erbB-2,[4,6] known to be associated with poor prognosis in breast tumors, is also evaluated in urothelial tumors.

In the literature, the frequency of c-erbB-2 overexpression ranges from 2% to 81%[4]. However, the uncertainty of the actual incidence of c-erbB-2 overexpression in these tumors is also addressed.[4]. Other studies emphasize that prognostic values may be contradictory after evaluating the data in many studies on the oncogene's predictive value[4,5,8].

This study aimed to reveal the relationship between clinical-pathological prognostic factors (e.g., histological tumor grade, pathological stage, lymph node, and distant organ metastasis) and survival time. This is also determining the frequency of immunohistochemically tested c-erbB-2 overexpression in patients with bladder urothelial carcinoma. In this way, c-erbB-2 overexpression or amplification can be defined as both and treatment option and a prognostic factor.

Materials and Methods

The design of this research is a retrospective study. Between January 2004 and February 2009, patients with bladder urothelial carcinoma diagnosed with pathology were investigated at the SBU Haydarpaşa Numune Training and Research Hospital of Pathology Laboratory. Cases monitored by oncology and urology outpatient clinics were selected from the cases with this diagnosis. Pathology reports and hematoxylin and eosin (H and E) preparations of the selected patients were re-examined. Cystectomy, cystoprostatectomy, and cystectomy-total abdominal hysterectomy resection specimens (n=41) diagnosed with classical urothelial carcinoma were included in the study. Cases undergoing transurethral resection, and urothelial tumor cases associated with specific variants of urothelial carcinomas or other tumor components in the pathological evaluation, were excluded from the study.

The study group consisted of patients with high histological grade, pathological stage pT2-pT4, advanced clinical-stage diagnosis, and follow-up of disease progression (n=41). Pathology reports and H and E preparations for the cases were re-examined. Age, gender, histological grade, pathological stage, lymph node, and distant organ metastases were evaluated and categorized. These patients' survival status was assessed by accessing the cases through outpatient records (26.8% excitus; 73.2% survive). Data on patients whose records could not be accessed were excluded from the study.

Immunohistochemical Analyses

Immunohistochemical analysis was conducted to determine the overexpression of c-erbB-2, one of the molecular subtypes of tumor cells. First, the preparation of slides and tissues was carried out by fixing the samples. Interpretation and analysis were performed following the reaction step involving c-erbB-2 antigen/antibody treatment.

H and E-stained preparation of the patients (n=41) was examined. At this stage, a paraffin block with a formalin fixation was applied where the tumor was dense. Then, 4-micron thick sections were taken on slides coated with Poly-L-Lysine. Deparaffinization was achieved by maintaining the sections overnight in an oven at 56. The process continued when the sections were immersed in xylene 3 times for 10 min and ethyl alcohol (96%; 80%; 70%) 3 times for 10 min. After the procedure, the slides were washed with distilled water. A solution of 10 mL of citrate buffer and six mL of distilled water was then used for antigen recovery. This solution was placed in a microwave oven and

boiled twice at 700 watts for 5 min and twice at 350 watts for 5 min.

The sections were removed from the oven and kept for about 20 min until they reached a room temperature of 22.4. The areas on the slides surroundings the sections were washed with distilled water drawn using a PAP Pen. This prevented the reagents from overflowing the section due to their hydrophobic properties. Then, hydrogen peroxide was dripped for 10 min onto the samples to block endogenous peroxidase. After washing, the samples were kept in this solution for 5 min with three separate phosphate buffer solutions (PBS).

Ultra V Block (Blocking Reagent-Ultra V Bloc, Labvision) was dripped onto the slides to form an antibody blockade. After 5 min, the solution was removed from the slide. Incubation with the c-erbB-2 primary antibody (CERB-B' Neomarkers, Clone e2-4001+3b5 Fremont California, USA) was achieved after 1 h at a room temperature of 22.4. The samples were kept in the PBS for 5 min. After washing three consecutive times with PBS, the samples were treated with a secondary antibody (Biotinylated Goat Anti-Polyvalent, Labvision) solution for 20 min and washed with PBS. After 20 min, streptavidin peroxidase (Labvision) was added. The samples were then rewashed with PBS and incubated with chromogen (UltraVision Large Volume Detection System AEC Substrate System) (RTU). Slides washed with distilled water were kept in a Mayer's Hematoxylin solution for 30 s; the opposite staining step was performed after a 15-min waiting period. Slides containing samples washed under tap water were covered with aqueous mounting.

Immunoreactivity Analysis

Breast carcinoma was used as an external positive control in the evaluation of the c-erbB-2 antibody. A membranous staining pattern was used for to determine c-erbB-2 positivity. The areas where staining were most intense and common in tumors were selected. Immunohistochemical staining was evaluated according to the staining criteria in the HercepTestTM score. This is a standard semi-quantitative immunohistochemical test accepted and approved by the U.S. Food and Drug Administration[9].

During the evaluations, a score of 0 represented no staining or staining in <10% of tumor cells. A score of 1+ represented incomplete membranous staining in more than 10% of tumor cells. A score of 2+ represented complete membranous staining of weak-medium intensity in more than 10% of tumor cells. A score of 3+ represented intense complete membranous staining in more than 10% of tumor cells. Scores of 0 and 1+ represented negative results, whereas scores of 2+ and 3+ were positive.

Statistical Analysis

NCSS Data Analysis 2008 (version 0007 and PASS) Statistical Software (Utah, USA) was used to analyze the obtained data. Descriptive statistical methods such as mean and standard deviation and comparison of quantitative data and intergroup comparisons of non-normally distributed parameters were performed using the Kruskal–Wallis test and Mann–Whitney U-test.

Chi-square test, the McNemar-Bowker test, and Kappa analysis were used to compare the qualitative data. The Kaplan–Meier analysis was used to perform survival analyses. The results were evaluated with a 95% confidence interval (95% CI), and the alpha significance level was considered to be<0.05.

Results

The patients' ages (n=41) in the study ranged between 47 and 85 years; the mean age was 66.63 years. Of the total, 90.2% were male and 9.8% were female (n=37). Of the total, 73.1% (n=30) underwent cystoprostatectomy, 22% (n=9) underwent cystectomy, and 4.9% (n=2) underwent combined total abdominal hysterectomy. All of the cases were of a high histological grade.

According to the primary tumor stage, 7.3% (n=3) of the cases were T2a, 24.4% (n=10) were T2b, 39% (n=16) were T3a, 7.3% (n=3) were T3b, and 22% (n=9) were T4a. Lymph node metastasis was not observed in 53.7% (n=22) of the cases when lymph node metastasis was examined (pN0). Of the total cases with lymph node metastasis, 19.5% (n=8) were in pN1 and 26.8% (n=11) were in pN2 stage.

After the Kaplan–Meier analysis, the follow-up period for the cases varied between 7 months and 5 years; the mean follow-up period was 4.12±1.52. The mean follow-up period for the 11 patients who did not survive was 1.72±0.78, and the mean follow-up period for the survivors (n=30) was s 5 years. Of the 41 cases in the study, 11 died. The cumulative survival rate was 73.2%, with a standard error of 6.9% for the period during which the most recent death occurred in the 3rd year of follow-up. The mean survival time was 4.12 years with a standard error of 0.23. C-erbB-2 was positive in 9.75% (n=4) of the cases and negative in 90.25% (n=37) of the cases. One case was 2+, and three cases were 3+ (Fig. 1).

Because all of the study group, cases were of high histological grade, all of the positive cases (n=4 cases with a score of 2+ or 3+) were of high histological grade. One case was

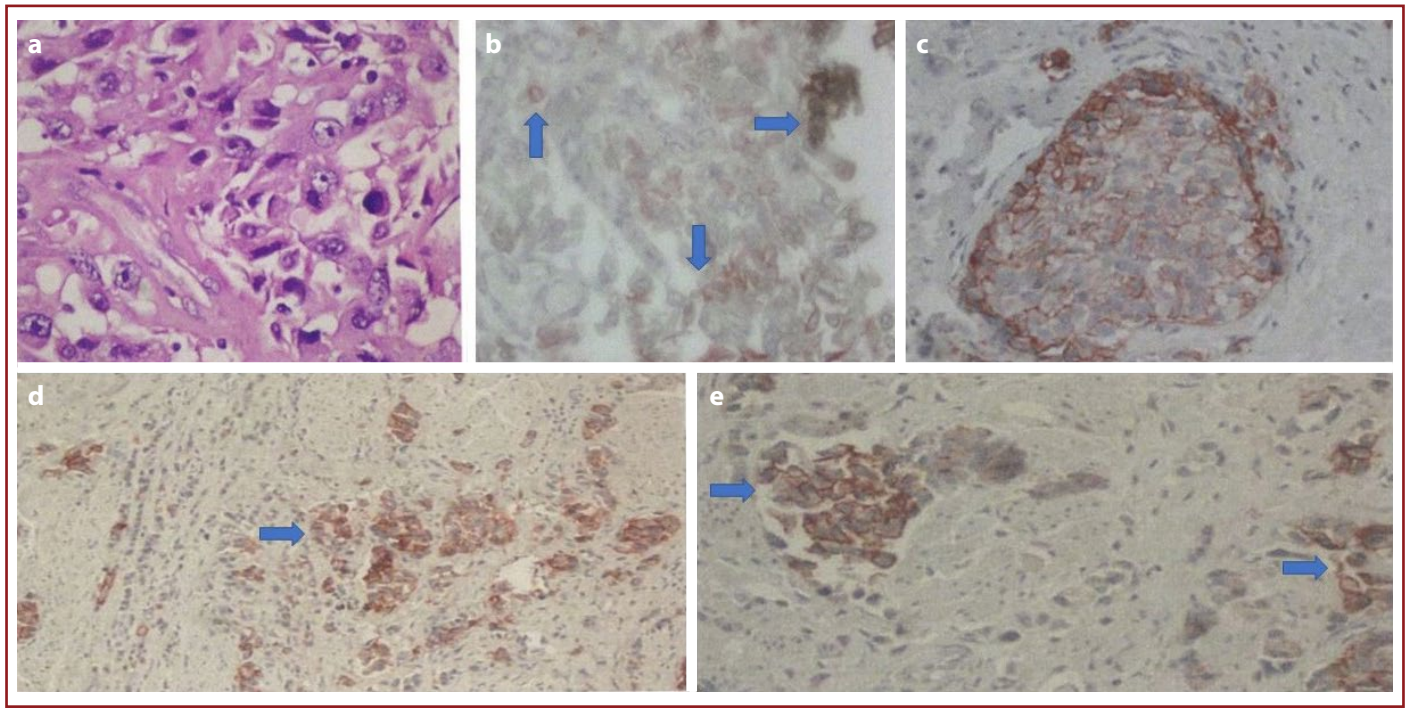

Figure 1. (a) Urothelial tumor of a high histological degree (H and E, ×400). **(b)** c-erbB-2 score of 1+ membranous staining and incomplete membranous staining in more than 10% of tumor cells (immunohistochemical examination, ×400). **(c)** c-erbB-2 score of 2+ membranous staining and complete membranous staining of weak-medium intensity in more than 10% of tumor cells (immunohistochemical examination, ×200). **(d)** c-erbB-2 score of 3+ membranous staining and intensive complete membranous staining in more than 10% of tumor cells (immunohistochemical examination, ×100). **(e)** c-erbB-2 score of 3+ membranous staining and intensive complete membranous staining in more than 10% of tumor cells (immunohistochemical examination, ×200).

found to be stage T2, one case stage T3, and two cases stage T4 compared to the pathological stage. Lymph node metastasis was not observed in any of the positive cases, whereas two cases were N1, and one case was N2 compared to regional lymph node metastasis. No distant organ metastasis was observed in any of the positive cases, and all patients were alive.

A statistical relationship between overexpression frequency and clinical-pathological prognostic factors and survival could not be evaluated. The number of positive cases with c-erbB-2 overexpression was low in the study group. However, it was notable that 75% of the positive cases were lymph node metastasis.

Discussion

The increase in c-erbB-2 overexpression and amplification in bladder urothelial tumors was first reported by Zhau et al.[10] in 1990. C-erbB-2 overexpression frequency is reported in an extensive range of 2–81% and at different rates in studies conducted to date[4]. In addition, the predictive value of c-erbB-2 overexpression is still not sufficiently elucidated and needs to be elaborated[11].

C-erbB-2 overexpression and amplification are also higher in advanced pathological stages such as high histological grades or pT2-T4 stages. In the literature, clinical-stage tumors are associated with an increased risk of metastasis or decreased life span[4,12].

In addition, some studies conclude that there is no relationship with histological grade, pathological-clinical stages, or prognostic significance[5,13]. It has been reported that it may be an important marker for predicting recurrence and/or progression of the disease in non-muscle-invasive urothelial cell carcinoma[14]. C-erbB-2 overexpression was 9.75% (n=4), even though the cases evaluated in this study consisted of high histological grade and advanced urothelial carcinomas. The results obtained were between 2% and 81%, consistent with the literature's research results. However, according to the rates provided in many studies,[3,4,11,12] our study results indicate lower rates.

The frequency of c-erbB-2 overexpression was 9.2% in a large series study consisting of 1005. The c-erbB-2 overexpression frequency was similar to those found in this study. In the literature, the frequency of c-erbB-2overexpression or gene amplification in urothelial tumors may have different results depending on the selected method (polymerase chain reaction, fluorescent in-situ hybridization, or

immunohistochemistry)[15]. This difference may be caused by application differences such as antigen retrieval and antibody used, in addition to methodological methods[15]. Scores of 2+ or 3+ were accepted positive threshold values. C-erbB-2 overexpression in the immunohistochemical examination is generally evaluated according to the American Society of Clinical Oncology/College of American Pathologists guidelines, as updated in 2013[16].

Various factors, such as intratumoral heterogeneity, different pathological and clinical stages of the cases selected in the studies, a lack of standardization in application and evaluation,[3,4,13,15,17] and ethnic[8] reasons may also contribute to the study results. The low frequency of c-erbB-2 overexpression in this study may be associated with a low number of patients, laboratory method used, antigen retrieval used, or intratumoral heterogeneity compared to the studies cited in the literature.

Bladder urothelial carcinomas are heterogeneous tumors with different morphological variants and molecular properties[18]. Various genomic and molecular classifications are currently made in bladder cancers consistent with information obtained about bladder cancer genetics. The cancer genome atlas (TCGA) divides bladder urothelial tumors into four main molecular clusters[19]. The c-erbB-2 mutation is located in the first and second clusters, expressing luminal breast and urothelial differentiation markers[19].

Yorozu et al.[20] reported that they detected significant mutation and amplification of c-erbB-2 gene in the luminal subtype in ureter and renal pelvis urothelial carcinomas. Certain morphological features may be associated with specific genetic changes, shown in colon and prostate cancers, in addition to molecular classifications[7]. The c-erbB-2 mutation has been reported at a high frequency in micropapillary urothelial tumors, a rare variant in urothelial tumors[7]. In addition, tumors showing amplification often show morphological heterogeneity and tumor-related chronic inflammation other than micropapillary structure[7]. Varying results related to the frequency of overexpression and amplification and prognosis reported in the literature suggest that the tumor may also be associated with the diversity in biological behaviors[4].

Currently, 131 patients with high-grade urothelial carcinoma are being evaluated in the study conducted by TCGA[8,19]. C-erbB-2 gene mutation and amplification were found in 9% of the study's patient group[8,19]. The fact that some of these molecular changes are similar to the molecular changes in breast tumors in TCGA suggests that both tumors have common molecular pathways[8,19].

Common molecular pathways are present in breast tumors and c-erbB-2 overexpression and amplification, demonstrating a higher amplification rate in micropapillary urothelial tumors. This represents a rare variant, indicating that anti-HER2 target treatment may be a possible treatment option in some of these tumors.

Positive results, such as a high response rate, were also promising for potential treatment options in these tumors. Those options could be applied in some phase-II clinical trials to use trastuzumab in combination with chemotherapeutic agents in bladder urothelial tumors[21].

In our study, the frequency of c-erbB-2 overexpression was 9.75%. This rate was lower than the rate obtained in many studies reported in the literature[3,4,11,12,22]. In our study, no statistical relationship could be established between histological grade, pathological stage, lymph node and distant organ metastases, and survival based on the low number of positive cases ($p>0.05$). This may represent a limitation in our research. However, the study's fundamental limitation is that the 41 patients evaluated reflected a low racial diversity.

Conclusion

The frequency of c-erbB-2 overexpression and amplification and its prognostic importance have varying and contradictory results in the literature. However, positive developments show that c-erbB-2 can be a possible prognostic factor and treatment option in bladder urothelial tumors. However, there is a need for multicenter large-scale research in which more patients of varying racial groups participate in a standardized laboratory application.

Ethics Committee Approval: Although this study is a retrospective study, ethics committee approval is not required.

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