Assessment of ERBB2 protein expression and gene amplification in urothelial bladder carcinoma by immunohistochemistry and fluorescence in situ hybridization

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Background: The goal of this study was to investigate ERBB2 (HER2) gene amplification and protein expression in bladder urothelial carcinoma. The HER2 status of 40 patients with transurethral resection or cystectomy was investigated. All cases were analyzed for both HER2 overexpression using immunohistochemistry and HER2 gene amplification using fluorescence in situ hybridization. Eighteen cases had a HER2 score-0 or score-1 (45%), 10 cases had a score-2 (25 %), and 12 cases had a score-3 (30%) with immunohistochemistry. HER2 gene amplification was observed in 16 out of 40 cases (40%). HER2 overexpression and gene amplification were correlated to a high histological grade (P< 0.0001), stage (P<0.0001) and 5-year survival (P<0.0001). A good correlation was observed between protein overexpression and gene amplification (p<0.0001).

Conclusion: Those data indicated that ERBB2 genetic abnormalities were associated with the prognosis of bladder cancer. Clinical assessment of ERBB2 amplification may represent an important factor for the development of personalized treatment programs for bladder urothelial cancer.

1. Introduction

Bladder cancer is one of the most common malignancies occurring worldwide (Jemal et al., 2008). Urothelial bladder carcinoma represents the first urological cancer (Charfi et al., 2013). In Egypt, according to the National cancer Institute (NCI), bladder cancer constitutes 30.3 % of all cancers (El-Bolkainy et al., 2008).

Several factors are involved in determining prognosis and treatment selection of urothelial carcinoma. Tumor grade and TNM stage are two independent factors which determine the treatment strategy (Charfi et al., 2013).

The identification of molecular targeted therapy is an important step to provide treatments that are better than systemic toxic chemotherapy (Sasaki et al., 2014).

Human epidermal growth factor receptor type 2 (HER2), also known as c-erbB-2 is located on chromosome 17q21 and is a transmembrane tyrosine kinase growth factor receptor (Olayioye et al., 2004). It consists of a growth factor binding ectodomain, a single transmembrane segment, an intracellular protein-tyrosine kinase catalytic domain, and a tyrosine-containing cytoplasmic tail. The genes for the four members of this family, HER1-HER4, are found on different human chromosomes. HER1-HER4 are associated with cell development, proliferation and differentiation, protein overexpression, and/or
gene amplification with a poor prognosis, rapid progression and metastasis in several types of carcinomas (Sasaki et al., 2014).

Overexpression of the human epidermal growth factor receptor 2 (ERBB2) (HER2), can lead to the activation of cellular signal transduction systems, resulting in the cellular transformation and cell proliferation events associated with cancer (Sebastian et al., 2006).

HER2 protein overexpression and gene amplification are observed in various malignancies, such as breast, ovary, stomach, colon, small intestine, and lung cancers (Takai et al., 2005, Bang et al., 2010, Wang et al. 2011, Gu et al., 2013, Sasaki et al., 2014). High expression level of ERBB2 has been significantly correlated with increased tumor invasion, metastasis, resistance to chemotherapy, and poor prognosis of those patients (Dent et al., 2007). However, a lack of reliable and accurate methods to assess the relationship between ERBB2 gene status and protein expression in bladder cancer has limited correlative assessments with clinical parameters, including survival and sensitivity to targeted agents.

The assessment of the HER2 status is crucial for the management of breast cancer, for both prognosis and prediction of the response to targeted therapies. HER2 protein overexpression and/or gene amplification are investigated routinely for almost all breast carcinomas and for chemotherapy-resistant advanced gastric cancers. The molecular targeted therapies for HER2 are performed in neoadjuvant, adjuvant and/or metastatic settings in both breast and gastric cancers, and the effects have been reported to be favorable (Bang et al., 2010, Dent et al., 2013).

There are many previous studies examining HER2 overexpression and/or gene amplification in urothelial carcinomas of the urinary bladder (Kolla et al., 2008, Bolenz et al., 2010 Charfi et al., 2013, Chen et al., 2013). Several reports on urothelial carcinomas have yielded conflicting results, with extensive variability in the incidence rates of HER2 overexpression, ranging between 0 and 89%, and HER2 gene amplification, ranging between 0 and 59% (Naruse et al., 2010, Olsson et al., 2012, Verdoorn et al., 2013). Therefore the present study is utilized fluorescence in situ hybridization (FISH) to assess amplification of the ERBB2 gene in bladder urothelial cancer patient samples. We combined this data with results from histopathological and immunohistochemical analyses to determine the relationship between ERBB2 expression status and clinicopathological variables in bladder urothelial cancer.

2. Materials and methods
2.1. Study population and tissue specimens
A series of 40 tumors from 40 patients (32 men and 8 women; mean age 66.12 ± 12.8 years, range 48–78 years) diagnosed with primary urothelial carcinoma of the urinary bladder was selected from the Pathology Department files, Faculty of Medicine, Benha University and National Cancer Institute, Cairo University, from March 2001 to April 2009. Paraffin-embedded tissues were obtained after transurethral resection for bladder tumor or cystectomy. Histopathological examination of hematoxylin–eosin (H&E) stained slides was carried out to confirm the diagnosis and determine the grade of urothelial cell carcinoma. The TNM classification was used for tumor staging and the 2004 WHO classification for tumor grading (Eble et al., 2004, Sobin et al., 2009).

2.2. Immunohistochemistry
Before immunostaining, Hematoxylin–eosin stained slides from the original paraffin-embedded blocks in each case were reviewed. Sections representing the most invasive areas of each tumor were selected. Briefly, 4-m-thick sections were cut from each paraffin block, mounted on poly-l-lysine coated slides, fixed in acetone for 10 min, and left to dry overnight at 37 °C. Slides were deparaffinized in xylene followed by absolute ethanol and subsequent rehydration in graded ethanol. The sections were then pre-treated with 3% hydrogen peroxide for 10 min to inactivate endogenous peroxides and washed in phosphate-buffered saline (PBS) solution. Heat-induced antigen retrieval was performed using epitope retrieval solution (DAKO) at 95 °C for 40 min. After heating, slides were allowed to cool down to room temperature and were briefly washed with PBS. Blocking solution (Protein Block Serum: 0.25% Casein in PBS containing Carrier Protein and NaN3; DAKO) was used for 5 min to prevent nonspecific immunostaining. Immunohistochemical staining was performed using the streptavidin-biotin peroxidase system. Slides were incubated for 30 min at room temperature with anti-HER2 (clone CB11, Novocastra, Newcastle upon Tyne, UK; dilution, 1/50), then washed with PBS before applying the biotinylated secondary antibody (anti-rabbit, DAKO) for 5 min. Sections were incubated with the streptavidin-biotin complex reagent (Universal Quick Kit, DAKO) for 15 min and developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 30 min. Finally, tissues sections were counterstained by Mayer’s hematoxylin, dehydrated, and mounted (DAKO). Internal/external, positive/negative controls for the antibody were used for validation of the reactions.

2.3. Evaluation of immunohistochemical staining
HER2 positivity was assessed using the ASCO scoring system, evaluating only membranous staining (Wolff et al., 2007). The level of HER2 protein expression was assessed semiquantitatively by the intensity and percentage of staining and scored on a scale of 0–3+. Only tumors with score 2+ and 3+ are categorized as positive c-erbB2 overexpression. A score of 1+ was defined as barely perceptible membrane staining in >10% of cells, a score of 2+ was defined as weak-to-moderate complete membrane staining present in >10% of tumor cells, and a score of 3+ was defined as strong complete membrane
staining in >30% of tumor cells. A cytoplasmic staining was considered nonspecific.

2.4 FISH analysis
ERBB2 gene amplification was analyzed by FISH using the Vysis Path Vysion ERBB2/DNA probe kit according to manufacturer’s instructions (Abbott Molecular, Abbott Park, IL, USA). Briefly, samples fixed in 10% formalin and embedded in paraffin were cut into 4 μm sections and incubated overnight at 56 °C. Slides were dewaxed in xylene and dehydrated in 100% alcohol for 5 min, followed by air drying. The slides were then incubated in proteinase K solution (0.2 mg/mL in 2 × SSC) at 37 °C for 15 min, washed with 2 × SSC (pH 7.0), and sequentially dehydrated in 70%, 85%, and 100% ethanol. After the application of 10 μl of probe to the target area of the slide, a coverslip was placed over the sample and sealed. Following denaturation at 73 °C for 5 min, the slides were allowed to hybridize overnight at 37 °C in a humidified chamber. After hybridization, the slides were washed in 0.4 × SSC containing 0.3% Nonidet P40 at 65 °C for 2 min and rinsed twice in 2 × SSC containing 0.1% Nonidet P40 for 2 min. The slides were then immersed in 70% ethanol for 3 min and dried at room temperature. After the slides were counterstained with 4’, 6-Diamidino-2-phenylindole dihydrochloride (DAPI), they were observed under a fluorescence microscope (Carl Zeiss, Goettingen, Germany) (Wang, 2010). ERBB2 levels were reported as ERBB2 gene: CEP 17 ratios in order to normalize values to the total number of chromosomes within each cell. Amplification was defined as an ERBB2 gene/CEP 17 ratios greater than or equal to 2.0 (Bang et al., 2010).

2.5. Statistical analysis
Data available for statistical evaluation was performed with Statistical Package for the Social Sciences SPSS (version 17.0, IBM, Chicago, IL, USA) for windows software. Initially, a descriptive analysis of all collected variables was performed. The correlation between expression and amplification of HER2 and tumor grade, TNM stage and 5-years survival were assessed with Fisher’s exact and chi-square tests. All differences were deemed significant at the level of p < 0.05.

3. Results
Table (1) showed expression of HER2 and its association with clinicopathologic characteristics of urothelial carcinoma studied.
Strong protein expression (3+) was observed in 12 of 40 tumors (30%) (Fig.1). An immunohistochemistry 0/1+ score was detected in 10 cases (25%) and 8 (20 %) cases respectively (Fig 3). Ten cases (25%) were scored as 2+ (Fig 2 and Fig 4). HER2 overexpression was significantly associated with the tumor grade (p< 0.001), stage (p<0.001) and 5-years survival (p<0.001) but not with age (p=0.269) or gender (p=0.758).
Ten cases (83.3%) out of cases exhibited score 3 and 5 cases (50%) out of the cases exhibited score 2 ERBB2 showed amplification of the gene by FISH
technique (Fig 5), however only one cases (5.6%) out of 0/1+ ERBB2 score cases. The correlation between ERBB2 expression and amplification was highly significant (P<0.0001) table (2). There was also a high significant correlation between amplification and the grade, stage and 5-year overall survival as all high grades and advanced stage cases showed ERBB-2 amplification (P<0.001). Fourteen cases out of sixteen cases (87.5%) died from the cancer bladder were associated with ERBB2 amplification (P< 0.0001).

Table (1): association of ERBB-2 protein expression and gene amplification with histopathological parameters of urothelial carcinoma cases studied.

<table>
<thead>
<tr>
<th>Clinicopathological Variables</th>
<th>ERBB-2 expression</th>
<th>P-value</th>
<th>ERBB-2 amplification</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-ve/1+</td>
<td></td>
<td>-ve</td>
<td></td>
</tr>
<tr>
<td>Age:</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&lt;70 years(n:14)</td>
<td>8(57%)</td>
<td>6(43%)</td>
<td>8(57.1%)</td>
<td>6(42.9%)</td>
</tr>
<tr>
<td>&gt;70 (n:26)</td>
<td>10(38.5%)</td>
<td>16(61.5%)</td>
<td>16(61.5%)</td>
<td>10(38.5%)</td>
</tr>
<tr>
<td>P</td>
<td>0.269</td>
<td></td>
<td>0.793</td>
<td></td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n:32)</td>
<td>14(43.7%)</td>
<td>18(56.3%)</td>
<td>19(59.4%)</td>
<td>13(40.6%)</td>
</tr>
<tr>
<td>Female(n:8)</td>
<td>4(50%)</td>
<td>4(50%)</td>
<td>5(62.5%)</td>
<td>3(37.5%)</td>
</tr>
<tr>
<td>P</td>
<td>0.758</td>
<td></td>
<td>0.876</td>
<td></td>
</tr>
<tr>
<td>Grade:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade(n:22)</td>
<td>17(77.3%)</td>
<td>5(22.7%)</td>
<td>20(90.9%)</td>
<td>2(9.1%)</td>
</tr>
<tr>
<td>High grade(n:18)</td>
<td>1(5.5%)</td>
<td>17(94.5%)</td>
<td>4(22.2%)</td>
<td>14(77.8%)</td>
</tr>
<tr>
<td>P</td>
<td>p&lt;0.001</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Stage:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTa + pT1(f:25)</td>
<td>17(68%)</td>
<td>8(32%)</td>
<td>20(80%)</td>
<td>5(20%)</td>
</tr>
<tr>
<td>≥pT2(f:15)</td>
<td>1(6.7%)</td>
<td>14(93.3%)</td>
<td>4(26.7%)</td>
<td>11(73.3%)</td>
</tr>
<tr>
<td>P</td>
<td>P&lt;0.001</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>5-years survival:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive(n:24)</td>
<td>16(66.7%)</td>
<td>8(33.3%)</td>
<td>23(95.8%)</td>
<td>1(4.2%)</td>
</tr>
<tr>
<td>Dead(n:16)</td>
<td>2(12.5%)</td>
<td>14(87.5%)</td>
<td>1(6.25%)</td>
<td>15(93.75%)</td>
</tr>
<tr>
<td>p</td>
<td>p&lt;0.001</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18(45%)</td>
<td>22(55%)</td>
<td>24(60%)</td>
<td>16(40%)</td>
</tr>
</tbody>
</table>

Table (2):ERBB2 analysis using immunohistochemistry and FISH in bladder cancer cases examined.

<table>
<thead>
<tr>
<th>ERBB-2 expression</th>
<th>ERBB-2 gene amplification</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>-ve/1+</td>
<td>17(94.4%)</td>
<td>1(5.6%)</td>
</tr>
<tr>
<td>2+</td>
<td>5(50%)</td>
<td>5(50%)</td>
</tr>
<tr>
<td>3+</td>
<td>2(16.7%)</td>
<td>10(83.3)</td>
</tr>
<tr>
<td>Total</td>
<td>24(60%)</td>
<td>16(40%)</td>
</tr>
</tbody>
</table>
Fig. 1. Strong and diffuse HER2 immunostaining surrounding the entire cell membrane (score 3+) (HER2 immunopeoxidase 100×).

Fig. 2. Moderate HER2 membranous immunostaining of high grade urothelial carcinoma (score 2+) (HER2 immunopeoxidase 200×).
Fig. 3. Weak and incomplete HER2 membranous immunostaining of urothelial cancer cells (score 1+) (HER2 immunostaining) (DAB ×200).

Fig. 4. Moderate HER2 immunostaining of low grade urothelial carcinoma (score 2+) (HER2 immunoperoxidase 100×).
Fig. 5. HER2 gene amplification in high grade bladder urothelial carcinoma analyzed by FISH. The green signal corresponds to chromosome 7, and the red signal corresponds to ERBB2 gene amplification is visible as red clustering (magnification, 1000 ×).
4. Discussion
In recent years, interest has grown in understanding the relationship between the biological characteristics of bladder cancer and the association of these characteristics with the clinical outcomes of the disease (Charfi et al., 2013). In recent years, new developments in cancer biology have led to the emergence of novel molecular-targeted therapeutics. These targeted drugs selectively act on cancer cells at the molecular, biochemical, and genetic levels, specifically targeting abnormal cells, with minimal effects on the function of normal cells. This phenomenon can lead to improvement of the efficacy of radiotherapy (Sasaki et al., 2014). Current studies have focused on understanding the molecular basis of bladder urothelial cancer in order to better choose effective treatment options. With the advent of novel targeted therapeutics, these molecular characteristics may be important for obtaining more effective therapeutic outcomes. Targeted therapy using trastuzumab is commonly applied worldwide for gastric and mammary carcinoma in preoperative (neoadjuvant), postoperative (adj-uvant) and metastatic settings (Sasaki et al., 2014).

ERBB2 is one of the HER family receptors. Little is known regarding the expression and function of these receptors in bladder cancer. Much knowledge regarding ERBB2 expression as well as its biological function in many cancers such as breast and gastric cancers is available (Duffy et al., 2005, Wang et al. 2011). Research techniques for characterization of ERBB2 expression have included immunohistochemistry (IHC), chromogenic in situ hybridization (CISH), and FISH.

Using IHC and FISH in the current study, ERBB2 protein expression and gene status have been analyzed in Egyptian bladder urothelial cancer patients in order to clarify its prognostic role in this bladder cancer. The ERBB2 protein was over-expressed (+2 and +3 score) in 55% (22/40) of urothelial carcinoma cases, and ERBB2 gene amplification occurred in 40% (16/40) of tumors. Of those cases that overexpressed ERBB2, ERBB2 gene amplification was present in 68.2% (15/22) of the cases. These results suggest that ERBB2 gene amplification is positively associated with ERBB2 protein overexpression.

IHC is currently the most widely-used method, and kits are commercially-available for detection of ERBB2. Application of IHC to evaluate the expression of ERBB2 in tumor cells has limitations, as the determination of staining is partially subjective. In contrast, results from the FISH are substantially less subjective than IHC, while maintaining sensitivity and specificity. Furthermore, the quantitative nature of FISH results can effectively reduce operator interference and inter-laboratory variations (Penault-Llorea, Cayre, 2004). In the present study, FISH revealed that ERBB2 gene amplification was present in 83.3% of cases with an IHC score of 3+, in 50% cases with an IHC score of 2+, and in only one case (5.6%) of cases with an IHC score of 1+. These results indicated that a statistically significant difference in ERBB2 gene amplification was present between the high (2+ and 3+) and low (1+) IHC score groups (P < 0.0001).
Several studies that employed immunohistochemistry to detect Her-2/Neu overexpression in urothelial carcinomas, have yielded a tremendous range of positive expression rates in contrast to studies based on gene amplification (Naik et al., 2011, El Gehani et al., 2012). A possible explanation is that, Her-2/Neu protein overexpression is believed to arise from a combination of two mechanisms. The first is gene amplification which is not a common mechanism in bladder cancer (Kruger et al., 2002). The second mechanism is upregulated transcription which reflects the nature of the Her-2/Neu protein as a growth factor. Therefore, higher levels of transcription factor, even in the absence of gene amplification result in increased Her-2/Neu protein expression (Ohta et al., 2001, Latif et al., 2004).

Several previous studies reported wide range of ERBB-2 expression in urothelial carcinoma ranging from 9.3% to 80% (Caner et al., 2008, Simonettiet al., 2009, Skagias et al., 2009, Bolenz et al., 2010, Charfi et al., 2013). Several hypotheses could explain these wide variations. One of the major issues is the variability in immunohistochemistry assays, related to the heterogeneity between kits, antibodies, protocols, interpretations of staining or cut-off values. Scoring systems are variable and there is no consensus on the definition of HER2 overexpression in bladder carcinoma. Most authors used criteria of Wolff et al. (2007), reported for breast carcinoma (ASCO scoring system).

In the current work, bladder cancer cases that scored 2+ and 3+ for HER2 were considered overexpressing HER2, however in a study carried by Charfi et al., (2013), only tumors scored 3+ were considered overexpressing HER2. They suggested that tumors scored 2+ should not be considered as overexpressing Her-2 without demonstration of HER2 gene amplification by in situ hybridization technique. Literature data showed that the majority of cases scored 2+ are not amplified by in situ hybridization. In a study carried by Laé et al., (2010), all cases scored 2+ were negative by fluorescent in situ hybridization (FISH) technique. However, Caner et al., (2008), noted that only 22% of cases were amplified by FISH and 33% of cases were amplified by real-time quantitative PCR for HER2 status. However, Naik et al., (2011), reported that cases scored as score-2 and score-3 as HER2 overexpression using only immunohistochemistry and treated them as HER2-positive cancers. On the other hand, in the current work, one case of the Her-2 0/1+ score was showed Her-2 amplification without overexpression of the Her2 protein. In a study carried out by Bang et al., (2010), they reported that cases showed HER2 amplification was determined as being HER2-negative cancer because they showed score-0 or score-1 with immunohistochemistry and concluded that this group (HER2 gene amplification but negative for HER2 protein overexpression) showed a poor response to trastuzumab in subgroup analysis in the ToGA trial. Also, Wolff et al., (2007) and Bang et al., (2010), suggested that HER2-positive cancers should be determined using a combination of immunohistochemistry and fluorescence in situ hybridization or DISH (dual-
color in situ hybridization) if the HER2 status is score-2 with immunohistochemistry for both breast and gastric cancers according to ASCO/CAP and ToGA recommendations.

For urothelial carcinomas, there are many papers reporting on the status of HER2 protein overexpression and gene amplification, but several studies have reported conflicting results. For example, the reported incidence of HER2 overexpression varies between 0 and 89%, and gene amplification between 0 and 59% (Olsson et al., 2012, Verdoorn et al., 2013). One of the reasons for this variation may be because of the characterization of special membrane staining, such as U-shaped HER2 in urothelial carcinoma and/or basolateral staining with immunohistochemistry. In the present study, several cases showed such immunostaining patterns, and these particular patterns are similar to those of gastric cancer (Wang et al., 2011), and are determined as being negative for HER2 because of the incomplete staining pattern under the previous ASCO/CAP criteria. This difficulty can be overcome by applying the same ToGA recommendations adopted for gastric cancer (Verschasselt-Crinquette et al., 2012).

In the present study both HER2 protein overexpression and gene amplification were significantly related to tumor grade, TNM stage, and 5-year survival. So ERBB2 status can promote progression and aggressive behavior of bladder urothelial cancer.

In previous studies, the prognostic significance of ERBB2expression in urinary bladder carcinoma is highly controversial. Thus, although series of studies (Korkolopoulou et al., 1997, Vollmer et al., 1998), have linked it to a better clinical outcome, others (Jimenez et al., 2001, Alexa et al., 2010), have found no prognostic association. However in several recent reports (Skagias et al., 2009, Enache et al., 2013, Shawky et al., 2013, Charfi et al., 2013), Her-2/Neu overexpression was associated with more aggressive clinical behavior of bladder carcinoma and was represented a prognostic factor for adverse disease outcome.

5. Conclusion
In conclusion, HER2 overexpression and amplification were strongly correlated with high tumor grade, stage and 5-year survival. There was a high correlation between EERBB2 expression and amplification. These data suggest a prognostic relevance of HER2 protein overexpression and amplification in patients with urothelial bladder carcinoma and indicate that there is a substantial collective of bladder carcinoma patients who might potentially profit from anti-HER2-directed therapy and underline the need for clinical trials.

6. References


42. Wang YK, Gao CF, Yun T, Chen Z, Zhang XW, Lv XX, Meng NL and Zhao WZ. Assessment of ERBB2 and EGFR gene amplification and protein expression in gastric carcinoma by immunohistochemistry and fluorescence in situ hybridization Molecular Cytogenetics 2011, 4:14
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