Potential for HER-2/neu molecular targeted therapy for invasive bladder carcinoma: Comparative study of immunohistochemistry and fluorescent in situ hybridization

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Received July 28, 2007; Accepted September 18, 2007

Abstract. Analysis of HER-2/neu expression in invasive bladder carcinoma was performed in order to evaluate the potential for molecular targeted therapy targeting HER-2. The subjects were 40 patients who were pathologically diagnosed with invasive transitional cell carcinoma of the bladder (pT2 to pT4). A Hercep test kit was used to detect HER-2 expression, and a Path Vysion kit was used for gene amplification. On immunohistochemical (IHC) staining, the primary tumors were HER-2 positive in 17 patients (17/40, 42.5%). According to the classification of grade, one Grade 2 patient (1/3) and 16 Grade 3 patients (16/37) were positive (P=0.99). According to the classification of stage, 12 pT2 patients (12/22, 54.5%), 2 pT3 patients (2/13, 15.3%), and 3 pT4 patients (3/5, 60%) were positive (P=0.55). Lymph node metastasis was found in 10 patients, and 3 pN2 patients were HER-2 positive (3/6, 50%) (P=0.32). A statistically significant difference was observed between HER-2-positive primary tumors and metastatic lymph nodes (P=0.02). In fluorescent in situ hybridization (FISH), HER-2/neu gene amplification was detected in the primary tumors in 5 patients (5/40, 12.5%). In all these patients, IHC staining was determined as 3+. Lymph node metastasis was found in 3 pN2 patients (3/6) (P=0.32), and in these patients with HER-2/neu gene-amplified metastatic lymph nodes, the primary tumors were also positive for gene amplification (P=0.02). In these cases, IHC staining was 3+ as well. The concordance rate of IHC-positive cases with cases positive for HER-2/neu gene amplification in FISH was 12.5% (5/40), and the concordance rate of IHC 3+ and gene amplification was 71%. This result suggests that, at present, patients who may potentially benefit from molecular targeted therapy targeting HER-2/neu for invasive bladder carcinoma should be identified by gene amplification analysis using FISH in IHC 3+ patients. In addition, it suggested that efficacy of molecular targeted therapy can be expected even for patients with metastatic lymph nodes as long as the primary tumors are positive for HER-2 expression.

Introduction

Currently, the standard treatment for invasive bladder cancer without metastasis is considered to be radical cystectomy. However, the results of radical cystectomy are substantially influenced by the pathological stage and the presence or absence of lymph node metastasis at the time of surgery. The cause-specific survival rate has been reported to be favorable at approximately 70% when invasion was observed only in the tunica muscularis (pT2), whereas the rate was 30-40% when invasion into the surrounding adipose tissue was observed (pT3), and approximately 20% when lymph node metastasis was noted (1,2). In addition, after radical cystectomy, most recurrences were distant metastases, only approximately 10% being local recurrence (3). Therefore, it is more important to eradicate such micro-metastases that cannot be identified by imaging rather than to increase local curability, to improve the treatment results of radical cystectomy. Surgical resection is not sufficient for invasive bladder cancer with involvement outside the bladder wall or lymph node metastasis, and additional therapy is required.

Multi-drug combination chemotherapy with anti-cancer agents is commonly used to treat progressive bladder cancer that is unresectable or metastatic. M-VAC therapy is a typical chemotherapy regimen and has been the gold standard since Sternberg et al (4,5) reported a response rate of 72% and complete remission rate of 36%. However, subsequent studies have revealed that the therapy has a low response rate with a short duration of response, which lowers the prospect of long-term survival; there are often concerns about dose intensity because a large proportion of the targeted patients are elderly, and the high toxicity of the drug is a substantial physical burden to those many elderly patients with bladder cancer (6,7). Moreover, at present, the standard treatment for M-VAC-resistant cancer has not been established. Therefore, the development of novel regimens replacing M-VAC therapy is...
Patients. This study included 40 patients who attended Aichi Medical University Hospital from January 2001 to December 2004, and were pathologically diagnosed with invasive bladder transitional cell carcinoma (pT2 to pT4). The diagnostic methods were radical cystectomy for 33 patients, and TUR-Bt for 7 patients. The patients were 47 to 80 years old (median, 65 years; mean, 65.6±8.4 years); 37 were male and 3 were female. The pathological grade was 2 and 3, in 3 and 37 patients, respectively. The stage was pT2, pT3 and pT4, in 29, 5, and 6 patients, respectively. The N classification was pN0, pN1, pN2 and pNx, in 23, 4, 6, and 7 patients, respectively.

Histopathological grade was based on the World Health Organization (WHO) classification (11), and the TNM classification (12) was used to evaluate primary tumors and lymph node metastases.

This study was approved by the Institutional Review Board at Aichi Medical University (no. 275), and every patient gave consent after being sufficiently informed, before HER-2/neu examination. Table I shows the characteristics of the 40 patients.

IHC method and evaluation. Paraffin sections, 5 μm thick, from bladder carcinoma samples taken at the operation were stained for HER-2 protein expression using a standardized Hercep test kit. The primary antibody in this assay is an affinity-purified rabbit polyclonal antiserum raised against an intracellular epitope of the human HER-2 molecule (Specification Sheet: K5205, Dako, USA). The staining procedure includes an antigen retrieval step consisting of a 40-min incubation in pH 6.0 citrate buffer (Target Retrieval, Dako) at 99°C in a water bath. Staining was performed using an automated staining apparatus for IHC (Autostainer, Dako) according to the manufacturer's guidelines. The slides were counterstained with haematoxylin. Each IHC experiment included a set of control slides. For the negative control, the primary antibody was replaced with an irrelevant, isotype-matched antibody to control for nonspecific binding of the secondary antibody reagent. The positive control slide consisted of sections of cell blocks of the three breast-cancer cell lines SKBR 3, MDA-MB-175, and MDA-MB-231, which express 2.4 million, 92,000 and 22,000 HER-2 receptor molecules by flow cytometry, respectively. These cells were included as a positive control. Tissue sections were stained for HER-2 protein expression using a standardized Hercep test kit (Vysis, Downers Grove, IL) was performed in order to evaluate primary tumors and lymph node metastases.

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FISH method and evaluation. In order to test whether amplification of the HER-2/neu gene occurred in bladder carcinoma, a FISH analysis was performed on a representative proportion of the tumors using the Path Vysion kit. The staining was performed on paraffin sections exactly according to the manufacturer's recommendations. Briefly, deparaffinized slides were treated with a protein-digesting enzyme at 37°C for 10 min. Preparations were denatured in 70% formamide/2X standard saline citrate (SSC), pH 7.0, at 72°C, for 5 min. Then, a solution containing the HER-2/neu probe coupled with Spectrum Orange and a specific probe for the centromere of chromosome 17 coupled with Spectrum Green were applied to the tissue sections at 37°C for 15 h. After hybridization, the unannealed probe was washed in 2X SSC/NP40 0.3% at 73°C for 2 min. Nuclei were counterstained using a 4,6-diamidino, 2-phenylindol (DAPI)/antifade solution, and stored in darkness at 8°C until evaluation. In each preparative run, positive controls (i.e. paraffin sections of breast cancers known to be amplified for the HER-2/neu gene or Probe Check control slides supplied by the manufacturer) were included. A Leica DMRB microscope equipped with appropriate filters for DAPI, Spectrum Green and Spectrum Orange was used to score the number of signals per nucleus. Images were captured.
using a Quantsics digital camera (Axioplan 2 imaging, Zeiss, Jena, Germany) and Quips FISH imaging software (Meta Cyte scanning image cytometer, Meta Systems, Altusheim, Germany). The number of fluorescent signals was counted in 60 nuclei of invasive tumor cells in two distant areas of the section for each case. For each tumor, the mean number of signals per nucleus was determined. Amplification was defined by at least a two-fold excess of the number of copies of the HER-2/neu gene with regard to the number of centromeres of chromosome 17 (Fig. 2).

For statistical analysis, Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 10.0 for Windows software was used. The chi-squared test was used to detect statistically significant differences between the groups, with a significance level of p<0.05.

Results

**IHC results.** HER-2 staining in primary tumors was 0 in 9 (22.5%) patients, 1+ in 14 (35%), 2+ in 10 (25%), and 3+ in 7 (17.5%). When 2+ and 3+ combined were considered HER-2 positive, 17 patients were HER-2 positive. According to the classification of grade, one (1/3, 33.3%) Grade 2 patient and 16 (16/37, 43.2%) Grade 3 patients were HER-2 positive. According to the classification of stage, 12 (12/22, 54.5%) pT2 patients, 2 (2/13, 15.3%) pT3 patients, and 3 (3/5, 60%) pT4 patients were HER-2 positive. Lymph node metastasis was found in 10 patients, and 3 (3/6, 50%) pT2 patients were HER-2 positive. No statistically significant difference was observed among all the classifications. However, there was a tendency for HER-2-positive patients to predominate in advanced-stage disease, although no statistically significant difference was observed (P=0.05) (Table II). In metastatic lymph nodes, HER-2 staining was determined as 0 in 7 (70%) patients and 3+ in 3 (30%), with no patient with 1+ or 2+. In addition, all primary tumors were Grade 3, and HER-2 staining was 3+ in all patients with positive lymph nodes. A statistically significant difference between patients with IHC-positive primary tumors and those with IHC-positive metastatic tumors was observed (P=0.02) (Table III).

**FISH results.** In primary tumors, the signal ratio was 0-1.0 in 4 patients (10.0%), 1.1-2.0 in 31 (77.5%), and ≥2.0, which was considered positive for HER-2/neu gene amplification, in 5 (12.5%). According to the classification of grade, all Grade 2 patients were negative for HER-2/neu gene amplification, but 5 Grade 3 patients were positive (5/37, 13.5%) (P=0.99). According to the classification of stage, 2 pT2 patients (2/22, 9%), 2 pT3 patients (2/13, 15.3%), and 1 pT4 patient were positive (1/5, 20%) (P=0.75). Lymph node metastasis was found in 10 patients, and 3 pN2 patients were positive for HER-2/neu gene amplification (3/6, 50%) (P=0.32). No statistically significant difference was observed among all the classifications (Table II). In all patients positive for gene amplification, IHC staining was 3+. The concordance rate of IHC 3+ patients and patients positive for gene amplification by FISH was 71% (5/7). Gene amplification was not detected in 2 patients although IHC staining was 3+ (Table IV).
metastatic lymph nodes, HER-2/neu gene amplification was 1.0-2.0 in 7 patients (70%) and ≥2.0 in 3 (30%), and the primary tumors were positive for HER-2/neu gene amplification as well, showing a statistically significant difference (P=0.02). These patients were similar to those who were 3+ by IHC staining (Table III).

Discussion

HER-2/neu (or HER-2) is a proto-oncogene located at chromosome 17q21, encoding a protein with a molecular weight of 185 kDa which has a transmembrane receptor structure. This protein is a tyrosine kinase-type cell surface receptor.
receptor which has amino acid sequences similar to those of an epidermal growth factor receptor, and whose cell differentiation and proliferation are stimulated by a ligand binding to an extracellular domain. Moreover, fundamental studies have shown that HER-2 overexpression induces cell transformation and that HER-2-positive tumors are more aggressive. With regard to the distribution of HER-2 in normal tissues, HER-2 is slightly expressed only in the liver, bile duct, gastrointestinal tract, skin, genital organs and urinary tract, with limited expression in most normal tissues (14-16). Therefore, the potential for molecular targeted therapy targeting HER-2 is of great interest.

Since Slamon et al (17) reported the correlation between HER-2 (c-erbB-2) gene amplification and breast cancer with a poor outcome in 1987, HER-2 has been considered to be a poor prognostic factor in breast cancer. In addition, breast cancer with HER-2 overexpression has been reported to be resistant to hormone therapy (18,19), resulting in establishment of treatment with trastuzumab (20). Currently, the methods to analyze HER-2 in tissues include analysis of gene amplification, mRNA overexpression, and protein overexpression; however, possible methods for use on formalin-fixed paraffin sections are IHC and FISH.

It has been indicated that examination of gene amplification rather than antigen expression is a more reliable method to identify patients with HER-2-positive breast cancer (21,22). However, it is inconclusive whether this result can similarly be applied to patients with cancer other than breast cancer, for example, patients with bladder cancer. Hence, we performed this analysis using both IHC staining and FISH in this study.

While HER-2/neu overexpression was observed in 12% (23) to 71% (24) of urothelial cancers, it correlated with grade and stage in some studies (25) but not in others (26,27), suggesting that this field has not yet been clarified. We studied patients with invasive bladder carcinoma in this analysis, and overexpression did not correlate with grade, stage or lymph node metastasis. In the literature on comparison between IHC and FISH, Sauter et al (28) reported that HER-2/neu gene amplification was detected in only 7% (10/141) of patients with lymph node metastases (n=10). FISH-A, FISH amplified; FISH-NA, FISH not amplified.

### Table II. Results of HER-2/neu overexpression (IHC) and gene amplification (FISH) by grade and stage.

<table>
<thead>
<tr>
<th>HER-2 positive</th>
<th>HER-2 negative</th>
<th>P-value</th>
<th>FISH-A</th>
<th>FISH-NA</th>
<th>P-value</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>1 (33.4%)</td>
<td>2 (66.6%)</td>
<td>0.99</td>
<td>0 (0%)</td>
<td>3 (100%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>G3</td>
<td>16 (43.2%)</td>
<td>21 (56.8%)</td>
<td></td>
<td>5 (13.5%)</td>
<td>32 (86.5%)</td>
<td></td>
</tr>
<tr>
<td>T classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>12 (54.5%)</td>
<td>10 (45.4%)</td>
<td>0.05</td>
<td>2 (9.1%)</td>
<td>20 (90.9%)</td>
<td>22</td>
</tr>
<tr>
<td>T3</td>
<td>2 (15.3%)</td>
<td>11 (84.6%)</td>
<td></td>
<td>2 (15.4%)</td>
<td>11 (84.6%)</td>
<td>0.75</td>
</tr>
<tr>
<td>T4</td>
<td>3 (60.0%)</td>
<td>2 (40.0%)</td>
<td></td>
<td>1 (20.0%)</td>
<td>4 (80.0%)</td>
<td>5</td>
</tr>
<tr>
<td>N classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>0 (0%)</td>
<td>4 (100%)</td>
<td>0.32</td>
<td>0 (0%)</td>
<td>4 (100%)</td>
<td>0.32</td>
</tr>
<tr>
<td>N2</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td></td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td>6</td>
</tr>
</tbody>
</table>

FISH-A, FISH amplified; FISH-NA, FISH not amplified.

### Table III. HER-2/neu overexpression (IHC) and gene amplification (FISH) in primary tumors and lymph node metastases.

<table>
<thead>
<tr>
<th>Primary tumors</th>
<th>FISH-A</th>
<th>FISH-NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH-A</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>HER-2 positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FISH-NA</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>HER-2 negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Patients with lymph node metastases (n=10). FISH-A, FISH amplified; FISH-NA, FISH not amplified.

### Table IV. Comparison of HER-2/neu between overexpression (IHC) and gene amplification (FISH).

<table>
<thead>
<tr>
<th>HER-2/neu overexpression (IHC)</th>
<th>0</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-2/neu (FISH)</td>
<td>NA</td>
<td>9</td>
<td>14</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>14</td>
<td>10</td>
<td>7</td>
<td>40</td>
</tr>
</tbody>
</table>

NA, FISH not amplified; A, FISH amplified.
with urothelial cancer (36 pTa, 42 pT1, 67 pT2-3/20 G1, 39 G2, 46 G3 and 6 with grade and stage unknown), whereas 43% (61/141) of tumors were HER-2 positive by IHC. In addition, Kruger et al (29) studied 203 patients with urothelial cancer and reported that 37% (76/203) of patients were HER-2 positive by IHC, whereas HER-2/neu gene amplification was detected in only 5% (2/42) of patients. Moreover, de Piniieux et al (30) reported that 23% (15/64) of patients with invasive urothelial cancer were HER-2 positive, while HER-2/neu gene amplification was detected in 28% (6/21) of patients. In our study, 42.5% (17/40) of patients were HER-2 positive by IHC, and 12.5% of patients were positive for gene amplification by FISH (5/40). In comparison of HER-2 expression between IHC and FISH, it was suggested that the dissociation between gene amplification and overexpression could be related to a point mutation that leads to protein overexpression, translocation or transcriptional upregulation (28). However, it is inconclusive whether this result can be applied to patients with bladder carcinoma, because the positive controls for the Hercep test kit for IHC are breast cancer cell lines. This could be a reason for the possible dissociation. Moreover, HER-2/neu expression is rare, and it may not be detected among extensive specimens on a glass slide in some cases because 400-fold magnification is required for observation by FISH, and because there may be a difference within the specimen slides as well. In addition, an automated analysis system may not be able to detect weak signals, and in such cases, analysis by fluorescence microscopy may also be necessary. Moreover, the dose effect caused by polysomy and DNA demethylation should be taken into consideration.

Our results suggest that performing IHC to identify IHC 3+ patients first, and then performing gene amplification using FISH on the immunohistochemically stained region may be effective to accurately diagnose HER-2/neu expression in patients with invasive bladder carcinoma.

Trastuzumab is a human monoclonal antibody against the outer cell membrane of the HER-2 receptor. By binding to the receptor, this drug blocks subsequent signals to the receptor, inhibits cancer growth, and produces an anti-tumor effect, representing a molecular targeted therapeutic drug targeting HER-2. It has been indicated that examination of gene amplification rather than antigen expression is a more reliable method to identify patients with HER-2-positive breast cancer (21,22) and our results suggested that 12.5% of patients with invasive bladder carcinoma may benefit from this drug. Moreover, it was suggested that patients suitable for this therapy should be identified by cost-effective IHC first, and then by performing gene amplification analysis using FISH in IHC 3+ patients. In addition, efficacy of this drug can be expected even in patients with lymph node metastases, as long as the primary foci are HER-2/neu positive. Further clinical studies in various phases are expected to establish the effectiveness of treatment with trastuzumab for bladder carcinoma.

References


