Potential for molecular targeted therapy of HER-2/neu for invasive bladder cancer: Examination of gene amplification by fluorescence *in situ* hybridization

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Abstract. Analysis of HER-2/neu gene amplification by fluorescence *in situ* hybridization was performed in 40 patients with invasive bladder cancer in order to evaluate the potential for molecular targeted therapy of HER-2 as a tailor-made treatment for patients with invasive bladder cancer. This study included 40 patients seen at the Aichi Medical University Hospital from January 2001 to December 2004 and were pathologically diagnosed with invasive transitional cell carcinoma of the bladder (pT2-pT4). The PathVysion kit was used to evaluate the status of HER-2/neu gene amplification, and a signal ratio ≥2.0 was considered positive for HER-2/neu gene amplification. In primary foci 5 patients (12.5%) were positive for HER-2/neu gene amplification. According to the classification of grade and stage, no statistically significant difference was observed. Lymph node metastasis was found in 10 patients, and 3 patients (30%) were positive for HER-2/neu gene amplification. In the patients with HER-2/neu gene-amplified metastatic lymph nodes, primary foci were also positive for gene amplification, showing a statistically significant difference. This study indicates that 12.5% of patients with invasive bladder cancer may benefit from molecular targeted therapy of HER-2, and that molecular targeted therapy can be expected to be effective even for patients with lymph node metastases as long as their primary foci are positive for HER-2/neu gene amplification.

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Introduction

Radical cystectomy is currently the standard treatment for invasive bladder cancer without metastasis. However, the results 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 about 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 around 20% when lymph node metastasis was noted (1-3). In addition, after radical cystectomy, most recurrences were distant metastases, only about 10% being local recurrence (4). 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* (5,6) 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 is often concern 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 the many elderly patients with bladder cancer (7,8). 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 on-going, and clinical trials of anti-cancer agents, mainly gemcitabine and taxane, are being conducted; however, their effectiveness is still under examination (9-11).

Drugs for molecular targeted therapies for cancer are being developed to target changes in genes and molecules that are characteristic of cancer. Conventional anti-cancer

agents primarily affect the process of nucleic acid, DNA, and microtubule synthesis, and exert anti-tumor effects. These agents, however, have limited effectiveness due to poor tumor selectivity, since they affect both normal and tumor cells. For conventional anti-cancer agents, therefore, the maximum tolerated dose without toxicity has been regarded as the optimal dose. However, molecular targeted therapeutic drugs generally have a weaker toxicity than anti-cancer agents. Moreover, these drugs can be administered safely even to elderly patients, which has been emphasized as an advantage. Furthermore, these drugs can be used in combination with existing anti-cancer agents, and are superior in that they can be used as a tailor-made treatment by coupling them with development of biomarkers.

In this study, we analyzed HER-2/neu gene amplification by fluorescence *in situ* hybridization (FISH) to determine the potential for molecular targeted therapy of HER-2 as an effective treatment for patients with invasive bladder cancer.

Patients and methods

Patients. We studied 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-pT4). The diagnostic methods were radical cystectomy for 33 patients, and TUR-Bt for 7 patients. The patients were aged 47-80 years (median 65, mean 65.6±8.4 years); 37 were male and 3 were female. The pathological grade was Grade 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 (12), and the TNM classification (13) 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 each patient gave an informed consent before FISH analysis. Table I shows the characteristics of the 40 patients.

Table I. Patient characteristics.

Patients no.	40
Age	47-80
Average age	65.6±8.35
Median age	65
Gender	
Male	37
Female	3
T classification	
pT2	22
pT3	13
pT4	5
N classification	
pN0	23
pN1	4
pN2	6
pNx	7
Grade	
G2	3
G3	37

Fluorescence in situ hybridization (FISH) analysis and evaluation method. 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 Food and Drug Administration (FDA)-approved kit, PathVysion (Vysis, Downers Grove, IL, USA). 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 SpectrumOrange and a specific probe for the centromere of chromosome 17 coupled with SpectrumGreen 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-diamidine, 2-phenylindol (DAPI)/ antifade solution, and stored in darkness at 8˚C until evaluation. In each preparative run, positive controls (paraffin sections of breast cancers known to be amplified for the HER-2/neu gene or ProbeCheck control slides supplied by the manufacturer) were included. A Leica DMRB microscope equipped with appropriate filters for DAPI, SpectrumGreen and SpectrumOrange was used to score the number of signals per nucleus. Images were captured using a Quantics digital camera (Axioplan 2 imaging, Zeiss, Jena, Germany) and Quips FISH imaging software (Meta Cyte scanning imaga cytometer, Meta Systems, Altlussheim, 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 2-fold excess of the number of copies of the HER-2/neu gene with regard to the number of centromeres of chromosome 17 (Fig. 1).

For statistical analysis, Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 10.0 for Windows software was used. The χ^2 test was used to detect statistically significant differences between the groups, with a significance level of $p<0.05$.

Results

In primary foci, 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

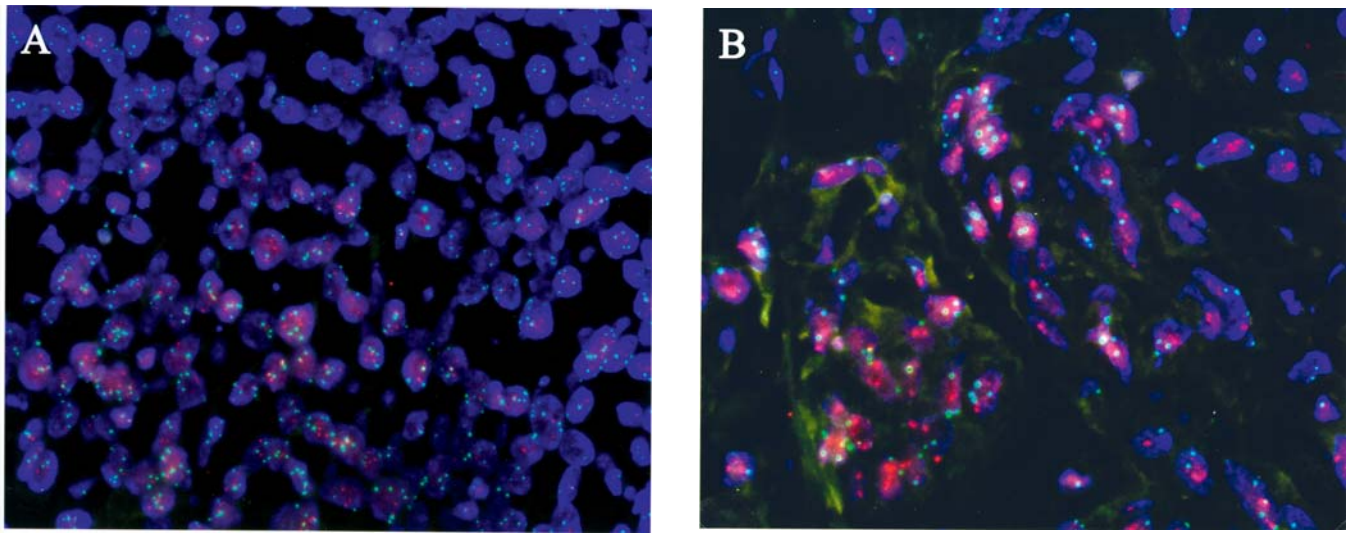

Figure 1. Fluorescence *in situ* hybridization of HER-2/neu on paraffin-embedded tissue sections (x400). A, Tumor without amplification. A mean number of two signals per nucleus was observed. B, Tumor with a high-level amplification.

Table II. Results of gene amplification by grade and stage.

	FISH-A (%)	FISH-NA (%)	Total	p-value
Grade				
G2	0 (0)	3 (100)	3	
G3	5 (13.5)	32 (86.4)	37	>0.99
T classification				
T2	2 (9)	20 (90.9)	22	
T3	2 (15.3)	11 (84.6)	13	
T4	1 (20.0)	4 (80.0)	5	0.75
N classification				
N1	0 (0)	4 (100)	4	
N2	3 (50)	3 (50)	6	0.32

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

Table III. Gene amplification in primary tumors and lymph node metastases.

	Primary tumors	
	FISH-A	FISH-NA
Lymph node metastases		
FISH-A	3	0
FISH-NA	0	7
Patients with lymph node metastases (n=10)		p=0.02

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

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 the groups (Table II). In metastatic lymph nodes, the signal ratio was 1.0-2.0 in 7 patients (70%) and ≥2.0 in 3 (30%), and primary foci were positive for HER-2/neu gene amplification as well, which showed a statistically significant difference (p=0.02) (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 which has amino acid sequences similar to those of an epidermal growth factor receptor, and that 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. 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 of 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 factor of poor prognosis 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.

Examination of gene amplification rather than antigen expression is a more reliable method to identify patients with HER-2-positive breast cancer (21,22). Hence, we performed this analysis of gene amplification by FISH using the FDA-recommended kit.

Up to 40 studies on HER-2/neu in urothelial cancer have been reported, including analysis by IHC. While HER-2/neu overexpression was observed in 12% (23) to 71% (24) of cancers in these studies, this overexpression was correlated with grade and stage in some studies (25,26) but not in others (27,28), suggesting that this field has not yet been established. In our patients with invasive bladder cancer, HER-2/neu overexpression was not correlated with grade, stage, or lymph node metastatic status.

Since accurate methods of determination of HER-2/neu expression in bladder cancer have not been established at present, analysis of HER-2/neu gene amplification by FISH, which has been established in identifying patients with HER-2/neu-positive breast cancer, was performed in this study.

In analysis of HER-2/neu gene amplification by FISH, De Pinieux *et al* (29) reported that HER-2/neu gene amplification was detected in 28.6% (6/21) of patients with urothelial cancer, while Sauter *et al* (30) reported that HER-2/neu gene amplification was detected only in 7% (10/141) of patients with urothelial cancer (36 pTa, 42 pT1, 67 pT2-T3/20 G1, 39 G2, 46 G3 and 6 with grade and stage unknown). In addition, Kruger *et al* (31) reported that HER-2/neu gene amplification was detected in 5% (2/42) of patients with invasive bladder cancer. In our study, HER-2/neu gene amplification was detected in 12.5% (5/40) of patients with urothelial cancer and in 30% (3/10) of patients with metastasis. The reason for such discrepancy in the frequency of HER-2/neu gene amplification detected by FISH is that HER-2/neu expression is rare among extensive specimens and it may not be detected 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.

There are few reports on HER-2/neu gene amplification in metastatic lymph nodes in bladder cancer. In analysis by IHC, Gardmark *et al* (32) reported that 40% (34/86) of metastatic lymph nodes were HER-2 positive and 72% of patients (34/47) also had HER-2-positive primary foci. In addition, Wester *et al* (33) reported that 67% (14/21) of metastatic lesions were HER-2 positive, and all patients with HER-2-positive metastatic foci also had positive primary foci. However, they also reported that 3 patients (14%) had negative metastatic foci despite having positive primary foci. Moreover, they reported that 89% (8/9) of patients with local metastases were HER-2 positive, while 50% (6/12) of patients with distant metastases were HER-2 positive, indicating that HER-2 expression tended to vary between local and distant metastases. This finding is interesting, and we consider that it should be examined in a larger number of patients. Our results also showed that 30% (3/10) of patients with lymph node metastasis were positive for HER-2/neu gene amplification, and in each of these 3 patients, primary foci were positive as well.

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 for HER-2. Our results suggested that 42.5% of patients with invasive bladder cancer may benefit from this drug, and that it can be expected to be effective for metastatic lymph nodes even in patients with lymph node metastases, as long as their primary tumors are HER-2 positive. Further clinical studies on various phases are needed to establish an effective treatment with trastuzumab for bladder cancer.

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