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ORIGINAL PAPER

No Strong Association Between HER-2/neu Protein Overexpression and Gene Amplification in High-grade Invasive Urothelial Carcinomas

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Abstract The generation of urothelial carcinoma is caused by the accumulation of various molecular changes, as in most malignancies. There are conflicting data about the status of HER-2/neu oncogene in urothelial carcinomas. The aim of this study was to determine the status of HER-2/neu oncogene in high-grade invasive urothelial carcinoma of urinary bladder both in protein and DNA level. We evaluated HER-2/neu protein overexpression by immunohistochemistry (IHC) and gene amplification by fluorescent *in situ* hybridization (FISH) and real-time quantitative PCR in paraffin-embedded samples of high-grade invasive urothelial carcinoma obtained from 36 patients. Polysomy 17 was also assessed by FISH. Immunohistochemically, HER-2/neu protein overexpression was observed in 22 (61.1%) tumors (ten tumors with score 3+ and 12 with score 2+). Fourteen of 36 tumors (38.9%) were evaluated as negative (score 0 or 1+). Complete concordance between FISH and the PCR was seen in all of the samples scored as 0 and 1+ by IHC. HER-2/neu gene amplification was observed in three of 27 (11.1%) tumors by FISH (nine samples were non-informative) and in eight of 36 (22.2%) tumors by the PCR. The complete concordance between HER2-2/neu protein overexpression and gene amplification was seen only in three of 27 tumors. Polysomy 17 was seen in nine tumors (33.3%). The results indicated that, in contrast to breast cancer, there was no strong association between HER-2/neu overexpression and gene amplification in invasive urothelial carcinomas, and polysomy 17 was higher in tumors showing HER-2/neu overexpression.

Keywords FISH · HER-2/neu · Immunohistochemistry · Real-time quantitative PCR urothelial carcinoma

Abbreviations

HER-2/*neu*	human epidermal growth factor receptor-2
IHC	immunohistochemistry
FISH	fluorescent *in situ* hybridization
The PCR	real-time quantitative polymerase chain reaction
FFPE	formalin-fixed paraffin-embedded tissues

Introduction

As in most other malignancies, the generation of urothelial carcinoma is caused by the accumulation of various molecular changes. Since the Food and Drug Administration approval of a novel therapeutic agent (Herceptin®) that directly targets human epidermal growth factor receptor-2 (HER-2/*neu*) protein for treatment of patients with metastatic breast carcinoma, the determination of HER-2/*neu* status has been an interesting matter for several studies focused on the changes in solid tumors [1–4].

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HER-2/*neu* is a proto-oncogene located at 17q21 that encodes a 185-kD transmembrane glycoprotein with tyrosine kinase activity. It has been reported that HER-2/*neu* overexpression was related to increase the rate of cell proliferation and angiogenic potential, and also to decrease the cell–cell adhesion. In bladder cancer, its amplification occurs predominantly in advanced and high-grade tumors, affecting approximately 10–20% of these cases [5, 6].

It is clinically important to determine the HER-2/*neu* protein overexpression and/or HER-2/*neu* gene amplification because clinical studies regarding the determination of tumor marker(s) focused on patient-based therapy (HER2—targeted therapy). Among the diagnostic techniques used, immunohistochemistry (IHC) is described as the best and easiest semi-quantitative method. However, there are some factors affecting the reproducibility of the method such as tumor fixation, antigen retrieval and usage of different antibodies having variable sensitivity and specificity. Several studies also suggested that IHC results yielding a 2+ score should be studied by another method, preferably fluorescent *in situ* hybridization (FISH) [7, 8].

FISH with dual-color hybridization is widely used for determining HER-2/*neu* gene copies in a cell, because it is easy to distinguish HER-2/*neu* amplification from chromosomal polyploidy. It is highly sensitive, specific, and standardized method although it is a time-consuming procedure and expensive procedure which also requires specialized equipment compared with IHC. The FDA has approved IHC for detecting HER-2/*neu* overexpression and FISH for quantifying HER-2/*neu* gene amplification [9]. Nucleic acid-based assays are used as alternative methods for quantification of HER-2/*neu* gene alteration since FISH has some disadvantages which can be reduced by using real-time quantitative PCR. Besides, target gene can be both amplified and detected simultaneously by the PCR in FFPE tumor tissues.

The aim of this study was to determine the status of HER-2/neu proto-oncogene in high-grade invasive urothelial carcinoma of urinary bladder both in protein and DNA level by using these three methods.

Materials and Methods

Tissue Samples

In the current study, we analyzed archival tumor samples of high-grade invasive urothelial carcinoma obtained from 36 patients (30 men and six women; mean age 68.53± 11.82 years, range 43 to 89) by transurethral resection performed at Pamukkale University Hospital between 1997 and 2005. Serial sections for IHC (5 μm), FISH (4 μm) and DNA isolation (5 μm) containing representative tumor tissue enriched for target cells were prepared.

Immunohistochemical Staining and Evaluation

In each case, the sections were stained immunohistochemically using monoclonal antibody against HER-2/neu protein (Clone CB11, Dako, USA) according to the manufacturer's instructions utilizing an automated staining system (NexEs IHC, Ventana, USA). In each staining run, positive and negative controls were included. Positive controls were FFPE sections of breast carcinoma that had been identified as scored 3+ while negative controls were performed by substituting primary antibody with phosphate-buffered saline.

All slides were analyzed independently by two pathologists and scored as 0 (no staining or <10% staining of tumor cells), 1+ (faint partial membrane staining in >10% of cells), 2+ (weak-to-moderate, complete membrane staining in >10% of tumor cells), and 3+ (strong, complete membrane staining in >10% of tumor cells).

FISH Analysis

The FDA-approved FISH assay, PathVysion (Vysis, Abbot Laboratories, IL, USA), was used for the identification and quantification of HER-2/*neu* gene alteration on FFPE sections fixed on slides according to manufacturer's recommendations. Briefly, the sections were deparaffinized in xylene and subjected to pretreatment including protease digestion for 20 min at 37°C following fixation with 10% buffered formalin and treatment with denaturation solution supplied in the kit. The prewarmed probe mixture containing the HER-2/*neu* DNA probe and the CEP 17 DNA probe was applied to the slides. After hybridization at 37°C for overnight (12–16 h), the slides were washed with post-hybridization wash buffer and counterstained with 0.2 μM 4,6-diamino-2-phenylindole (DAPI). Slides were preserved at −20°C in the dark before signal enumeration. A minimum of 60 tumor cell nuclei was analyzed using a Nikon E-600 fluorescence microscope (Nikon, Japan) equipped with DAPI, yellow and red–green bandpass filters.

Amplification of the HER-2/*neu* was evaluated at a ratio of 2.0 or greater (according to the manufacturer's recommendations) and then all cases were classified as either positive or negative. The relative increase in HER-2/*neu* copy number was determined when there were more HER-2/*neu* signals than CEP 17 signals in >17% of cells and HER-2/*neu*: CEP 17 ratio ≥1.04 including HER-2/*neu* gene amplification. Polysomy 17 was defined as a mean CEP 17 count of 3.0 or higher [10].

Real-time Quantitative PCR Analysis of HER-2/neu Amplification

For real-time quantitative PCR, DNA samples were extracted from FFPE tissues using the QIAamp DNA Mini Kit (Qiagen Inc., USA) as described by the manufacturer and stored at 4°C until use as template. The PCR was performed with the "LightCycler HER-2/*neu* DNA quantification kit" (Roche Diagnostics, Germany). A 112-bp fragment of the HER-2/*neu* and a 133-bp fragment of the reference gene, gastrin, were amplified during the PCR. The final results were calculated as a ratio of HER-2/*neu* to the reference gene copies in the sample, which were normalized with a ratio of HER-2/*neu* to the reference gene copies in the calibrator DNA. According to the manufacturer, a two-fold increased ratio (≥2) should be regarded as positive for HER-2/*neu* DNA amplification.

Results

Immunohistochemical investigation of HER-2/neu protein overexpression in 36 tumors revealed score of 3+ in ten tumors (27.8%) and score f 2+ in 12 tumors (33.3%). Fourteen tumors (38.9%) were negative (scored as 0 or 1+) (Fig. 1).

The results of HER-2/*neu* gene amplification analysis by FISH or real-time quantitative PCR associated with and without HER-2/*neu* overexpression are summarized in Tables 1 and 2, respectively. FISH was successful for HER-2/*neu* in 27 of 36 tumors (75.0%) (Fig. 2). Nine non-informative cases (three in IHC 0–1+, three in IHC 2+, and three in IHC 3+) were repeated for three times at least, however hybridization was not observed in any of the tumors probably because of the preanalytical variables such as fixation and age of tumor tissues. Three out of 27 (11.1%) tumors were positive for HER-2/*neu* gene amplification. One of them was scored as 3+ while the others were scored as 2+. Of the tumors analyzed, nine (three IHC 0–1+, four IHC 2+, and two IHC3+) (33.3%) were polysomic for chromosome 17 copy number (including cases with gene amplification).

HER-2/*neu* gene amplification was found in 22.2% (eight of 36) of all tumors by the PCR. The median amplification ratio was 3.4 (range 2.19–6.12) in tumors with gene amplification. Four of eight samples (50.0%) classified as positive by the PCR were scored as 3+, and four of those (50.0%) were scored as 2+. None of the tumors scored as 0–1+ by IHC were found to be amplified by the PCR.

All of the tumors scored as negative (0–1+) for HER-2/*neu* overexpression by IHC were also negative for HER-2/*neu* gene amplification by FISH and the PCR. Among the tumors which were positive by IHC (scored as 2+ and 3+ tumors), the complete concordance between HER2-2/neu overexpression and gene amplification was seen only in three of 27 tumors. Among 11 tumors with HER-2/neu overexpression, there was no correlation between the results of IHC and the results of FISH and the PCR for gene amplification (Fig. 3).

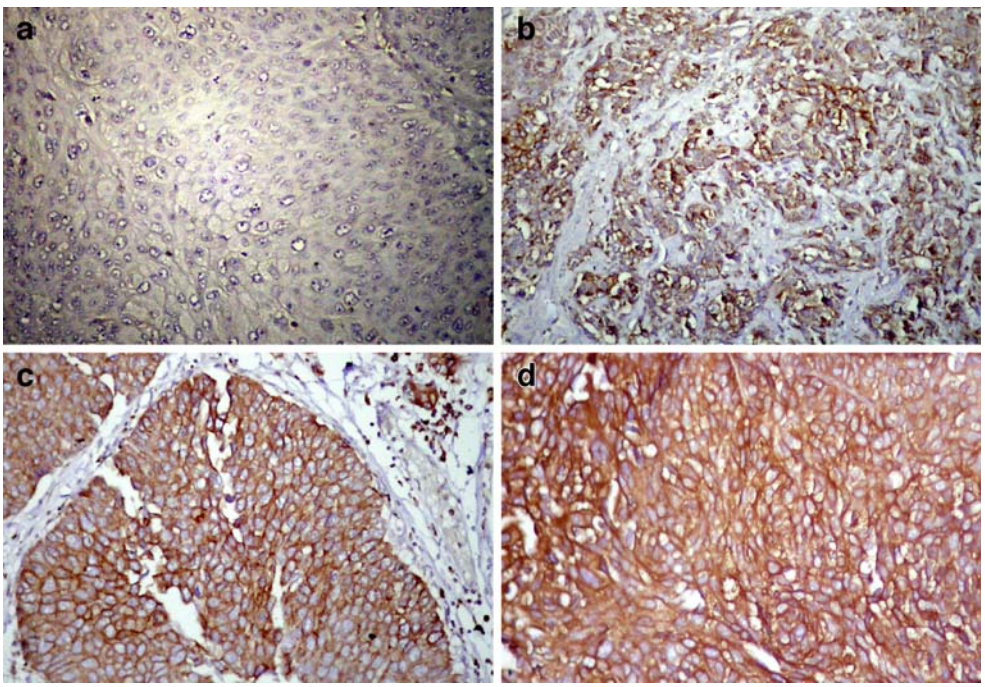

Fig. 1 Comparison of HER-2/neu IHC staining in high-grade urothelial carcinomas. HER-2/neu expression scored as 0 and 1+ (=negative) (**a** and **b**, respectively), 2+ score: weak-to-moderate complete membrane staining in >10% of tumor cells (**c**), 3+ score: strong complete membrane staining in more than 10% of tumor cells (**d**), original magnification ×200

Table 1 Comparison of immunohistochemistry (IHC) and fluorescent *in situ* hybridization (FISH) for HER-2/*neu* status in 27 bladder tumors

FISH	IHC 3+	IHC 2+	IHC 1+ and 0	Total (%)
HER-2/*neu* gene amplification	1	2	0	3 (11.1)
HER-2/*neu* non-gene amplification	6	7	11	24 (88.9)
Total	7	9	11	27 (100.0)

Discussion

The aim of this study was to investigate the relationship between immunohistochemically detected protein overexpression and gene amplification examined by FISH and the PCR in high grade invasive urothelial carcinoma of the urinary bladder. By using the standard scoring system described in the "Materials and Methods" section, 33.3% and 27.8% of the samples reported in the study were scored as 2+ and 3+ by IHC, respectively. Edwards et al. [11] have reported 71% of high-grade urothelial carcinomas showed strong HER-2/*neu* protein overexpression while in another study done with same subset of the carcinomas, the overexpression has been observed in 41% of the tumors [12]. This heterogeneity is probably due to the differences in antibodies, protocols, and interpretation. The results of this study is in accordance with those obtained in previous studies and confirm the considerably heterogeneity of HER-2/*neu* protein overexpression in high-grade urothelial carcinomas [13–15].

It is well known that HER-2/*neu* gene amplification occurs predominantly in advanced and high-grade invasive urothelial carcinomas, affecting approximately 10–20% of these cases [15, 16]. In this study, HER-2/*neu* gene alteration was determined with both FISH and real-time quantitative PCR. Informative results by FISH were obtained in 75% of the carcinomas. Simon et al. [15] have reported that FISH analysis was successful for HER-2/*neu* in 1,465 of 1,853 (79.1%) bladder tumors. This may be due to several factors including age of tumors, thermal and mechanical damage of tissue samples occurring during transurethral removal as in discussed in several reports. [15, 17, 18]. In this study, HER-2/*neu* gene amplification was only identified in 11.1% of the tumor samples by FISH. These positive samples were also positive by the PCR and positive for polysomy 17. Nine (33.3%) of high-grade urothelial carcinomas were found positive for polysomy 17

Table 2 Comparison of immunohistochemistry (IHC) and real-time quantitative PCR for HER-2/*neu* status in 36 bladder tumors

Real-time quantitative PCR	IHC 3+	IHC 2+	IHC 1+ and 0	Total (%)
HER-2/*neu* gene amplification	4	4	0	8 (22.2)
HER-2/*neu* non-gene amplification	6	8	14	28 (77.8)
Total	10	12	14	36 (100.0)

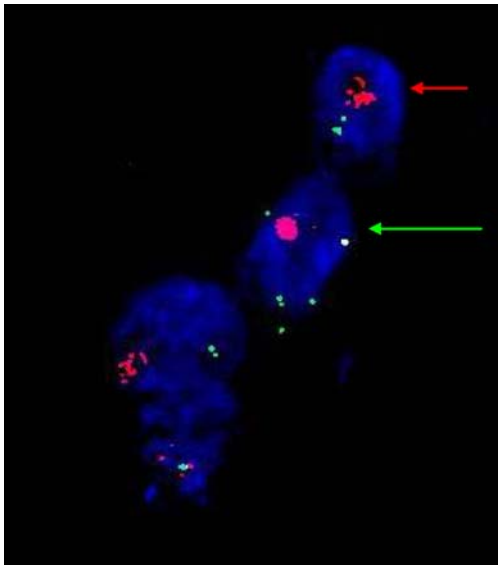

Fig. 2 *Her-2/neu* amplification determined by FISH, ×1,000. Heterogenous amplification of *Her-2/neu* in a tumor sample showing a tumor cell with amplification (*clusters of red signals*) and diploidy of chromosome 17 (*green signals*) (*red arrow*, *upper part*) and a tumor cell with amplification and polysomy 17 (*green arrow*, *lower part*)

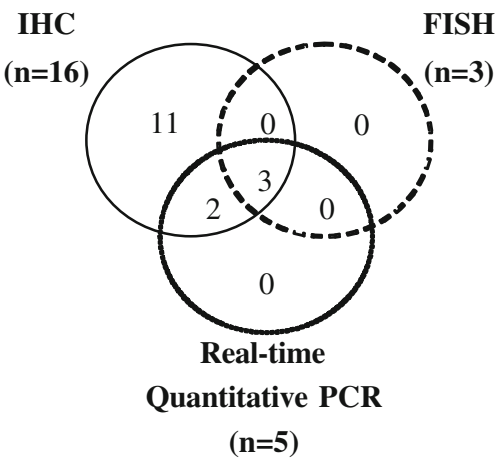

Fig. 3 Venn diagram showing the concordance between FISH and real-time quantitative PCR among the 16 tumors determined as positive for HER-2/neu overexpression (scored as 2+ and 3+). Six tumors (three tumors scored 2+ and three tumors scored 3+) were non-informative by FISH and were not included in the diagram. The *solid, dashed, and dotted ellipses* describe the results of IHC, FISH, and real-time quantitative PCR, respectively. The complete concordance between three methods was only seen in three tumors while two tumors were positive by IHC and the PCR. Of the 16 tumors, 11 with HER-2/neu overexpression were determined as negative for gene amplification by both FISH and the PCR

and seven of those were positive both by IHC and the PCR. Two out of the carcinomas with polysomy 17 were negative for HER-2/*neu* protein overexpression, and similar results were also reported in breast cancers [19, 20]. In the previous literature, the rate of polysomy 17 has varied and riched up to 65.5% in bladder tumors [16, 21, 22]. Hovey et al. [23] have reported that polysomy 17 was one of the most common numerical aberrations (23.9%) in bladder cancers. It is well known that the abnormalities in chromosome 9 copy number is associated with non-invasive urothelial carcinomas while those in chromosome 17, especially polysomy 17 is associated with the invasive carcinomas. These results suggested that the increase of chromosome 17 copy number was associated with aggressive bladder cancer and might play a role in tumor progression. However, while the effect of gene amplification on HER-2/*neu* protein overexpression has been well documented, the impact of polysomy 17 on HER-2/*neu* overexpression without gene amplification has not been explained. Kelly et al. [24] suggested that increased HER-2/*neu* gene copy number resulting from polysomy 17 alone was unlikely to significantly contribute to HER-2/*neu* gene amplification at the level of transcription in invasive breast cancers with polysomy 17. These data need to be confirmed by a larger study if it is also possible or due to an unidentified mechanism(s) at the posttranslational level for high-grade urothelial carcinomas.

It is possible to get informative results for DNA with small size by real-time quantitative PCR although DNA undergoes substantial changes during formalin-fixation. In this study, 22.2% of the carcinomas were found as positive for HER-2/*neu* gene amplification by the PCR. Miyamoto et al. [25] focused on HER-2/*neu* gene amplification in different grades and stages of bladder carcinomas by using semiquantitative PCR with a different control gene and found that HER-2/*neu* gene amplification was positive in 12 (57%) of 21 high-grade tumors. This difference may be due to diversity in methodology and samples used. There is no report related with investigating HER-2/*neu* gene amplification by real-time quantitative PCR and determining any concordance between both of these two methods in high-grade invasive urothelial carcinomas according to our knowledge. The results of this study suggested that real-time quantitative PCR may possess a high potential to determine HER-2/*neu* gene amplification in high-grade invasive urothelial carcinomas as similarly in breast cancers. Taken all together, molecular studies that focus on determining HER-2/*neu* gene amplification with different methods based on DNA and/or RNA in high-grade urothelial carcinomas are necessary to confirm the data.

In this study, HER-2/*neu* protein overexpression, HER-2/*neu* gene amplification and the increase of chromosome 17 copy number in high-grade invasive urothelial carcinomas were identified. No strong association between HER-2/*neu* overexpression and HER-2/*neu* gene amplification was observed in the urothelial carcinomas in contrast to breast cancer. Therefore molecular diagnostics for *HER-2/neu* amplification might be more probably necessary to select patients who are most likely respond to anti-HER-2/neu therapy. Besides, there was a good correlation between polysomy 17 and HER-2/neu overexpression. Although the sample size was limited, the data was in accordance with those obtained in previous studies. This study suggests that one or several mechanisms other than gene amplification may be responsible for HER-2/neu overexpression in a considerable fraction of high-grade urothelial carcinomas, and polysomy 17 might also play a role in tumor progression.

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