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HER2/neu gene amplification and protein overexpression in G3 pT2 transitional cell carcinoma of the bladder: a role for anti-HER2 therapy?

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Abstract

HER2/neu is an oncogene encoding a type 1 tyrosine kinase growth factor receptor. Polysomy 17, gene amplification and HER2/neu protein overexpression are associated with a poor prognosis in transitional cell carcinomas (TCC) of the bladder. Due to the application of different laboratory techniques, the exact incidence of *HER/neu* abnormalities remains uncertain in TCC. Standardised laboratory techniques are therefore important in the determination of the *HER2/neu* status if an assessment of the potential value of anti-*HER2/neu* treatments in the clinical management of patients with TCC is to be made. In this study, 75 TCCs with evidence of detrusor muscle invasion at first clinical presentation were included. Gene amplification, polysomy 17 and *HER2* copy number were assessed using fluorescence *in situ* hybridisation (FISH), with separate probes for chromosome 17 and *HER2/neu*. Protein overexpression was assessed using immunohistochemistry (IHC), with the CB11 antibody and a scoring system evaluating only membranous staining as positive. The mean patient age was 69.5 years (range 42–93 years) and the median survival was 15 months (range 1–156 months). Polysomy 17 occurred in 97%, increased *HER/neu* copy number in 92% and *HER2/neu* gene amplification in 7%. Protein overexpression occurred in 57% of cases. Polysomy 17 and HER2/neu protein overexpression are common in G3 pT2 TCCs of the bladder. However, gene amplification is uncommon. Mechanisms other than gene amplification may be responsible for protein overexpression in this tumour type. Evidence from breast cancer suggests that only tumours with *HER2/neu* gene amplification respond to the anti-*HER2/neu* therapy trastuzumab (Herceptin™). If this were true for bladder cancer, only 4/75 (5%) of G3 pT2 TCCs would be suitable for treatment. The role of trastuzumab in these tumours remains untested at present.

Keywords: *HER2/neu*; Herceptin™; Trastuzumab; Bladder cancer; Gene amplification; Protein expression; TCC

1. Introduction

Whilst approximately 10–15% of 'superficial' (stage pTa/Ti) transitional cell carcinomas (TCC) of the urinary bladder progress to detrusor muscle invasion (stage pT2+), the majority of pT2+ tumours are not associated with earlier 'superficial' disease. The prognosis of patients with pT2+ tumours is considerably worse than superficial tumours, since up to 50% of these patients already possess micro-metastatic disease at diagnosis [1].

It has been suggested that pT2 tumours have acquired a greater number of genetic changes than pTa/Ti tumours, and these changes may be important in the development of a malignant phenotype [2]. Polysomy 17 in TCC occurs frequently, is independent of tumour polyploidy and is more common in high-grade tumours compared with low-grade tumours [3]. Li and colleagues reported polysomy 17 in 0% of grade 1 tumours, 30% of grade 2 and 80% of grade 3 tumours [4].

The *HER2/neu* oncogene is located on chromosome 17 and encodes for a tyrosine-kinase growth factor receptor [5,6]. Protein overexpression and gene amplification of *HER2/neu* occur more commonly in pT2 tumours compared with pTa/Ti tumours and are associated with a poorer prognosis [7–12]. It is also evident that there is a wide variation in the reported incidences

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of gene amplification (4–59%) and protein overexpression (21–89%), possibly due to the differences in laboratory methodology used in these studies. Standardised methodology now exists for assessment of *HER2/neu* in human breast tumours [13]. Gene amplification is assessed with fluorescence *in situ* hybridisation (FISH) and protein overexpression with specific antibodies and scoring systems. This is of particular importance because anti-*HER2/neu* therapies are now in use to clinically treat tumours that overexpress *HER2/neu*. For example, trastuzumab (Herceptin™, Genetech Inc. San Francisco, USA) is a monoclonal antibody targeted against the HER2/neu receptor and response rates of 50% (when used with chemotherapuetic agents) and an increased time to disease progression have been observed in the treatment of breast cancers amplified for *HER2* [14]. Furthermore, trastuzumab acts synergistically, in breast cancer, with agents more commonly used in the treatment of TCCs such as cisplatin, with minimal additional toxic side-effects, suggesting it may be of value in the treatment of TCC [15].

In the current study, *HER2/neu* gene amplification, polysomy 17 and *HER2/neu* copy number was assessed with FISH and protein overexpression using the CB11 monoclonal antibody in 75 TCCs (all G3 pT2). The relevance of these findings to the possible use of trastuzumab in the treatment of TCC is discussed below.

2. Patients and methods

2.1. Patients

Tumours with evidence of muscle invasion were identified from a bladder cancer database in the Department of Surgery, Glasgow Royal Infirmary. All patients had a full clinical follow-up (age, date of diagnosis, cystoscopic follow-up, tumour stage and grade, and survival). Five-micron sections of formalin-fixed paraffin tissue were cut onto sialinised slides and baked at 56 °C overnight. All representative TCCs analysed had one section stained with haematoxylin and eosin, and were re-staged and re-graded by a specialist urological pathologist. Ethical approval was obtained from the Local Research Ethics Committees of the relevant hospitals for this study.

2.2. *Fluorescence* in situ *hybridisation (FISH)*

The FISH methodology was followed as outlined: tissue sections were dewaxed and rehydrated, then subject to pretreatment with: 0.2 N HCl for 20 min at room temperature, 8% sodium thiosulphate at 80 °C for 30 min, and 0.5% pepsin in 0.01 N HCl for 26 min at 37 °C. Tissue sections were postfixed in 10% neutral buffered formalin at room temperature for 10 min before dehydration in ascending grades of alcohol and air drying. These steps were carried out on a VP2000 robotic pretreatment slide processor (Vysis, UK, Ltd). The tissue sections were assessed for the extent of tissue digestion [16]. Tissue sections were denatured in 70% formamide. 2×SSC, pH 7–8 at 72 °C for 5 min on the Omnislide hybridisation module (Hybaid, UK, Ltd). Probes for the pericentromeric region of chromosome 17 (SpectrumGreen™) and the locus-specific probe for HER2 (SpectrumOrange™) were used. For each section, 1 μl of each probe was added to 7 μl hybridisation mix (50% formamide, 2×SSC, 10% dextran sulphate) and 1 μl deionised water and denatured in a water bath at 72 °C for 5 min and then added to the slide and hybridised overnight at 37 °C. Posthybridisation washes were in 0.4×SSC, 0.3% Nonidet 30, pH 7, at 72 °C for 2 min. The sections were mounted in 0.25 μg ml^{-1} DAPI antifade (Veactashield, UK) and viewed with a Leica DMLB microscope. A triple band pass filter block spanning the excitation and emission wavelengths of the SpectrumOrange™ and SpectrumGreen™ and diamidino, 2 phenyl indole 2 hydrochloride (DAPI) was used in the analysis of the hybridisation. Image capture was achieved using a digital camera (Leica DC 200 Leica, UK).

2.3. *FISH scoring*

Serially sectioned haematoxylin and eosin (H&E) stained tissue sections were first examined to localise areas of TCC. FISH sections were then scanned at ×400 magnification to localise the areas of interest. In total, three areas were identified and in each area 20 nuclei were assessed. Chromosome 17 copy number and *HER2* copy number were assessed for each of the 20 nuclei at ×l000 magnification. An average chromosome 17 copy number and *HER2/neu* copy number was obtained by totalling the number of signals over the 60 nuclei and dividing by the number of signals. Control sections of normal bladder and *HER2/neu* gene amplified breast tumours were included in each run. Values for disomy were derived from the analysis of normal bladder postmortem tissue, as previously assessed in Refs. [3,16]. The average *HER2/neu* copy number was 1.7 (±0.1) and hence a *HER2/neu* copy number greater than 2 (1.7 + 3×standard deviation (S.D.)) was defined as 'increased'. The average chromosome 17 copy number was 1.7 (±0.06) and hence a polysomy 17 was defined as a chromosome 17 copy number greater than 1.88 (1.7 + 3×S.D.). Gene amplification was defined as a *HER2*/chromosome 17 ratio of greater than 2 [3], based on the value used in breast cancer diagnostics.

2.4. *Immunohistochemistry*

Antigen retrieval was performed by placing the slides in a pressure cooker containing 1 l of boiling water with

0.37 g (1 mM) of ethylenediamine tetracetic acid (EDTA). The pressure cooker was placed in a microwave (850 W) for 13.5 min, then the lid was removed and the slides left to stand for another 20 min. The slides were then loaded onto an automated machine (NEXUS II, Ventana, USA), with a rotating slide carousel. The following reagents were added in sequence automatically by the machine (all steps were performed at 37 °C, and reagents were purchased prepacked): (1) 0.1 ml of inhibitor (containing 1.1% hydrogen peroxide, to block endogenous peroxidase) for 4 min, (2) 100 μl (0.63 μg/ml) of CB11 monoclonal primary antibody (IgG 1), (3) 0.1 ml each of amplifier A (Ig G heavy and light chains) and B (Ig G heavy chains) for 8 min. (4) 0.1 ml of biotinylated secondary antibody (Ig G) for 8 min, (5) 0.1 ml of avidin– horseradish peroxidase (HRPO) conjugate, which binds to the biotin, for 8 min, (6) 0.1 ml of diaminobenzidine (DAB) for 8 min, (7) 0.1 ml of copper sulphate to enhance the brown precipitate, (8) 0.1 ml of haematoxylin and 0.1 ml of bluing agent containing lithium carbonate to stain the nuclei blue. The slides were then dehydrated through graded alcohols and mounted with DPX. Normal bladder tissue controls and breast cancers with *HER2/neu* gene amplification and strong protein overexpression were used as controls in each run.

2.5. Scoring

Representative areas were identified from the H&E sections and corresponding areas were scored using a conventional light microscope. Only membranous staining was scored, with cytoplasmic staining being ignored. A four-point scale was used: '0' if there was no membranous staining, '1' if there was weak membranous staining in at least 10% of cells, '2' if there was moderate membrane staining in at least 10% of cells, '3' if there was strong membranous staining in at least 10% of cells.

3. Results

The clinical stage of the tumour was not used, but instead all tumours had to demonstrate unequivocal detrusor muscle invasion on re-staging and re-grading by the uropathologist. As such, all 75 tumours were grade 3 and stage pT2. There were 54 males and 21 females, with a mean age of 69.5 years (range 42–93 years). 22 patients were still alive at the time of data collection. Of the 53 patients who died, 30 died with metastatic bladder cancer (nuclear bone scan was positive), 23 of locally advanced disease. 48 patients received radical radiotherapy and 15 had palliative radiotherapy and 12 had no other adjuvant therapy. The median survival was 15 months (range 1–156 months).

The mean tumour *HER2/neu* copy number was 3.42±S.D. (range 1.73–18.10) and 69/75 cases (92%) had an increased *HER2/neu* copy number compared with normal tissue (Fig. 1). The mean chromosome 17 number was 3.14±S.D. (range 1.81–12.51) and 73/75 (97%) of the tumours exhibited polysomy 17 (Fig. 2). A scatterplot of *HER2 /neu* copy number versus chromosome 17 copy number is given in Fig. 3, illustrating the close correlation.

However, only 5/75 (7%) of the tumours displayed *HER2/neu* gene amplification (HER2/chromosome 17 ratios of 4.05, 2.21, 2.5, 2.55 and 2.74, respectively). The mean *HER2/neu*:chromosome 17 ratio number used to calculate gene amplification was 1.14 (range 0.62–4.05). Examples of disomic, polysomic and gene amplified tumours are given in Fig. 4(a–c).

Following evaluation of immunohistochemical staining, 11 tumours (11/75, 15%) were scored as '2+' and 32 (32/75, 43%) as '3+'. Overall, 57% of the tumours were evaluated as displaying HER2/neu protein overexpression. Of the tumours that scored as 'negative' for immunohistochemistry, 21 (21/75, 28%) were scored

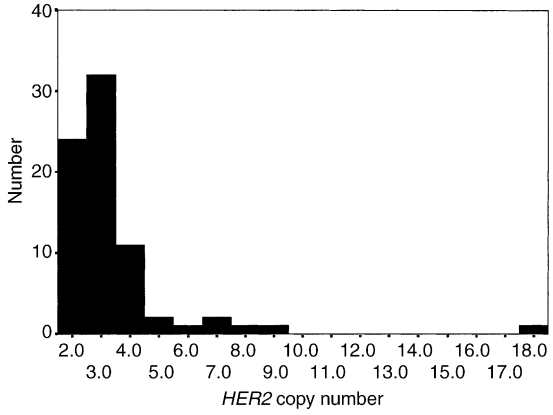

Fig. 1. Histogram of *HER2* copy number in the 75 tumours. Values greater than 2.0 have increased *HER2* copy number. Hence, 92% have increased *HER2* copy number.

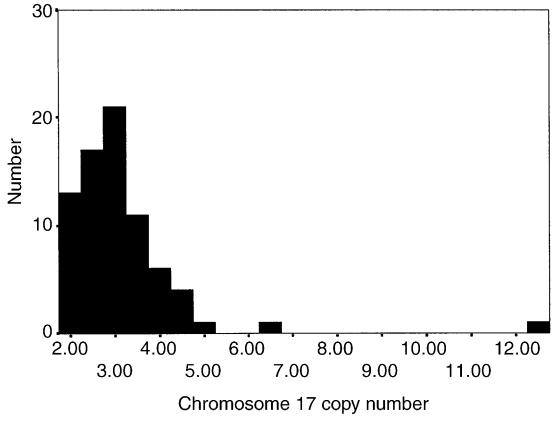

Fig. 2. Histogram showing the distribution of polysomy 17 in the 75 tumours. A chromosome 17 value greater than 1.88 is considered polysomic. Hence, 97% of tumours had polysomy 17.

as '0' and 11(11/75, 15%) as '1+'. Examples of tumours staining as '2+' and '3+' are given in Fig. 5(a–b) along with tumours scored as '0' and '1+' (c–d) for comparison. Four of the five tumours with *HER2/neu* gene amplification also strongly overexpressed the HER2 protein ('3+') and one did not ('1+'). These results are summarised in Table 1.

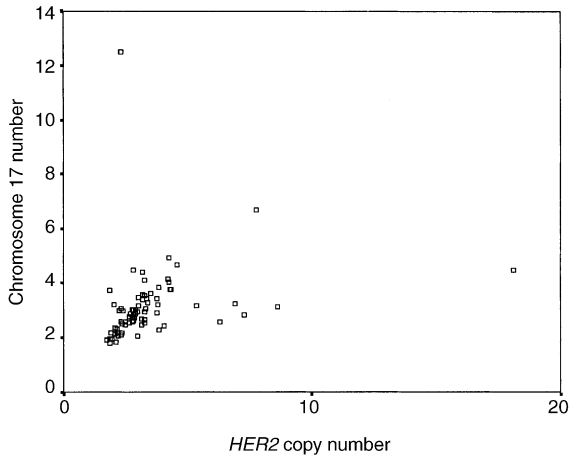

Fig. 3. Scatterp lot of *HER2* copy number versus chromosome 17 copy number.

4. Discussion

Polysomy of chromosome 17 in TCC of the urinary bladder occurs independently of tumour polyploidy, suggesting that it is a chromosome-specific event [17]. As detailed in Table 2, polysomy 17 is more common in muscle-invasive TCCs, compared with non-muscle-invasive tumours [4,16]. Hence, polysomy 17 might be an important genetic event in the development of muscle-invasive bladder cancer. The *HER2/neu* oncogene is located on chromosome 17, and encodes a type II transmembrane tyrosine kinase growth factor receptor. Overexpression of *HER2/neu* is associated with an increased cell-proliferation rate, increased angiogenic potential and reduced cell-to-cell adhesion [5,6].

Previous studies have suggested that *HER2/neu* gene amplification rates in TCC are low, in comparison with

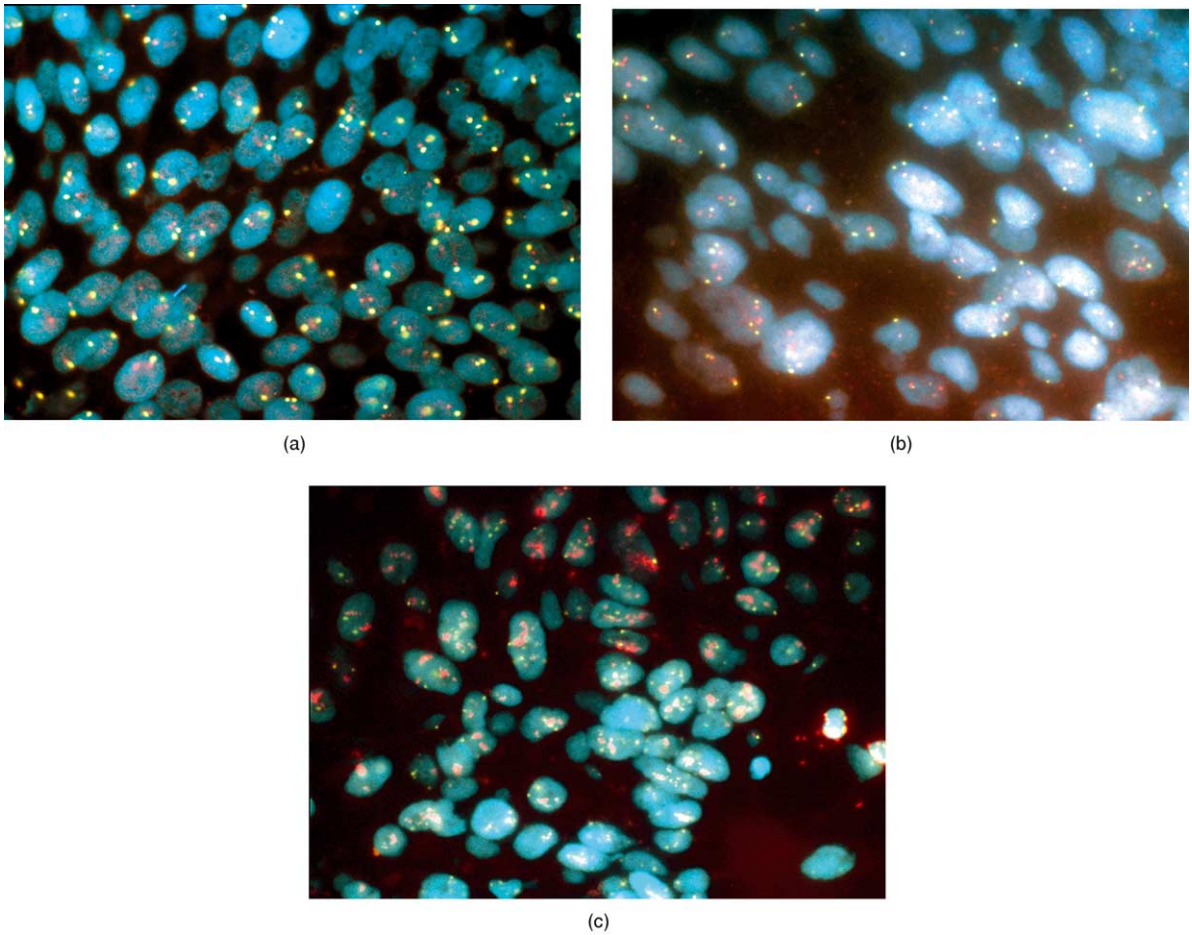

Fig. 4. (a) This shows a fluorescence *in situ* hybridisation (FISH) image with evidence of disomy of chromosome 17 and *HER2* copy number. (b) This shows a FISH image with evidence of polysomy of chromosome 17 and *HER2* copy number. (c) This shows a FISH image with evidence of gene amplification of *HER2* (a *HER2*/chromosome 17 ratio of more than 2).

polysomy *HER2* and protein overexpression rates [4,7–12,16]. Mellon and colleagues used Southern blotting with a radiolabelled cDNA probe to assess gene amplification and found gene amplification in 1/24 (4%) of tumours [7]. Miyamoto and collegues and Underwood and colleagues both used semiquantitative polymerase chain reaction (PCR) and found gene amplification in 32 and 9% of the tumours, respectively [8,12]. Miyamoto and colleagues used thymidine kinase (136 base pairs) and creatine kinase (125 base pairs) as the two control fragments, and defined gene amplification if there were more than four copies of the *HER2* PCR fragments (219 base pairs) relative to the control fragments. In order to prevent false-positive results due to the preferential amplification of shorter PCR fragments, Underwood and colleagues used an analytical algorithm, a 82-base pair control gene (*IFN-γ*) with a 92-base pair *HER2* reference gene, as well as cell lines with known

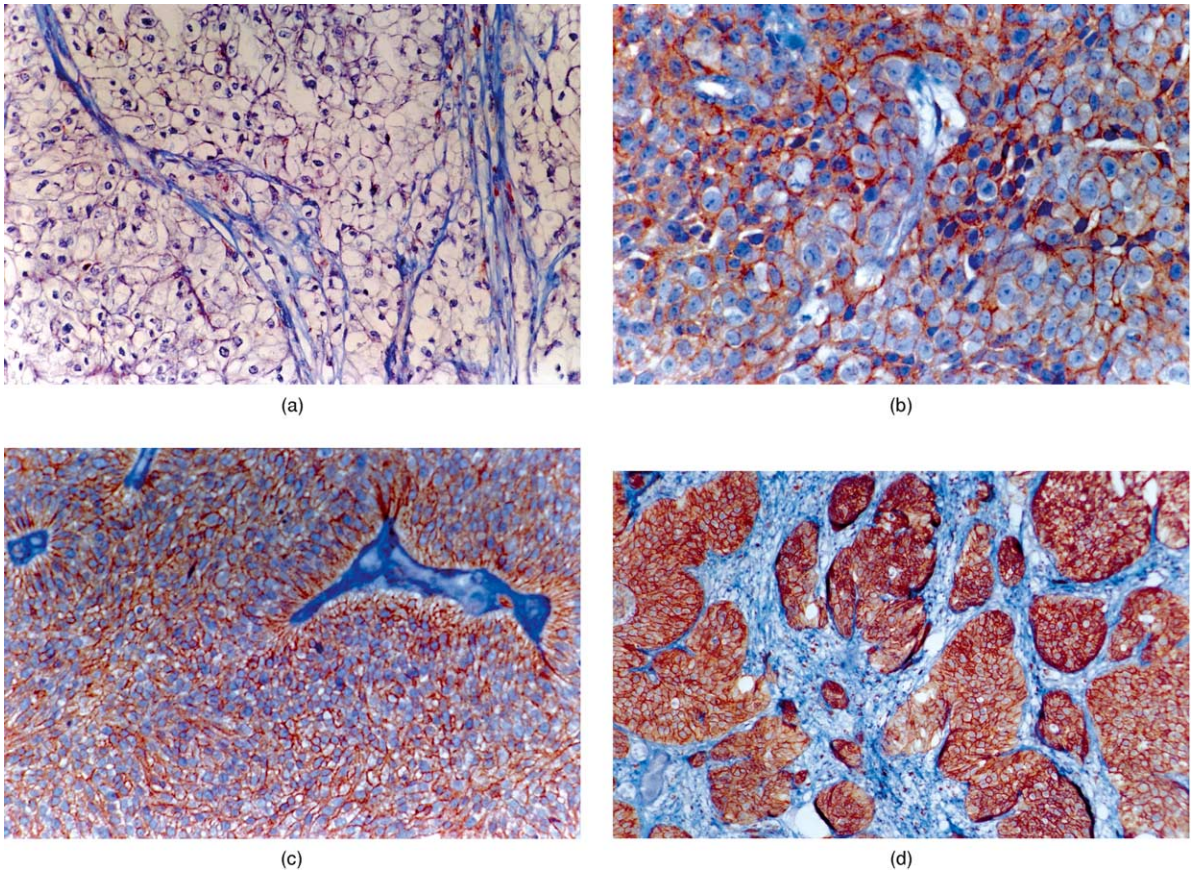

Fig. 5. (a) This shows an immunohystochemical (IHC) image with evidence of strong membrane staining in at least 10% of cells—scored as a '3+'. (b). This shows an IHC image with evidence of moderate membrane staining in at least 10% of cells—scored as a '2+'. (c) This shows an IHC image with evidence of minimal membrane staining in at least 10% of cells—scored as a '1+'. (d) This shows an IHC image with no membrane staining present at the cell membrane—scored as a '0'.

Table 1
Polysomy 17, *HER2* copy number, gene amplification and IHC results

Parameter	Mean	Range	% Increased *HER2*, polysomy 17, gene amplification or protein overexpression
HER2 copy number	3.42	1.73–18.1	92
Chromosome 17	3.15	1.81–12.51	97
HER2/chromosome 17 ratio >2	1.14	0.62–4.05	7
Protein overexpression (2+ or 3+)			2+15
			3+43

IHC, immunohistochemistry.
Note the high rates of polysomy 17 and increased *HER2* copy number in comparison with the low gene amplification rates. Protein overexpression (2+, 3+) rates were also higher than gene amplification suggesting that other mechanisms are responsible for protein overexpression.

HER2 copy numbers to act as controls. Copy numbers of HER2 that were more than twice the level of the control genes were defined as amplified. With the application of such stringent laboratory techniques, the overall gene amplification rate was 9% [12]. This is similar to the 9% reported by Sauter and colleagues using FISH, which is the method approved for *HER2* testing in breast cancers [10,13]. It is therefore apparent that the laboratory techniques in all these studies are different, as well as different definitions of gene amplification. However, in all the studies (Table 3), the level of gene amplification is higher in the tumours with muscle invasion compared with 'superficial' tumours. Using dual colour probes for chromosome 17 and *HER2/neu*, and defining gene amplification as a *HER2/neu* copy number as greater than that of chromosome 17, Fukushi and colleagues observed gene amplification in 29% of ovarian carcinomas [18]. In contrast, the incidence of polysomy 17 was significantly higher at 73%. In tumour cell lines established from ovarian carcinomas, HER2/neu protein overexpression rates using monoclonal antibodies have been observed in up to 50% of cases [19]. In prostate cancer, using IHC with the Herceptest, only 8% of cancers overexpress the *HER2* protein [20]. In the same study, using dual-colour FISH and defining gene amplification as a *HER2/neu* ratio of more than 1, Lara and colleagues observed no gene amplification, although only seven of the 62 tumours were assessed in this study. Other authors have reported a higher rate of protein overexpression with the progression of hormone-dependent (27%) to hormone-resistant disease (78%, [21]). The present study comprised 75 tumours with definite evidence of detrusor muscle invasion at first clinical presentation, all assessed with FISH, and, as such, represents one of the largest studies of its kind in the literature to date. In this study, we observed an extremely high incidence of polysomy for chromosome 17 (97%) and concordant increases in the *HER2/neu* copy number (92%) in most of the tumours. In contrast, the observed incidence of *HER2/neu* gene amplification was low (7%). These results suggest that polysomy 17 occurs commonly in these highly aggressive tumours. This is consistent with previous studies, suggesting that polysomy 17 is more frequent in high-grade tumours compared with low-grade tumours (Table 2). We have also previously shown that polysomy 17 is related to both recurrence and progression of superficial TCCs [16]. In particular, high copy numbers for chromosome 17 (>3–4 copies per cell) appeared to be associated with pT2 + disease. Polysomy 17 has previously been shown to be a chromosome-specific event, independent

Table 2
Incidence of polysomy 17 in TCC

	Watters and colleagues, 2000 [16]	Li and colleagues, 1998 [4]
G1	4/47 (9%)	0/5 (0%)
G2		10/31 (32%)
G3	33/76 (43%)	18/23 (78%)
pTa-T1	20/101 (20%)	12/38 (32%)
pT2-T4	14/22 (64%)	15/21 (71%)

TCC,transitinal cell carcinomas
Association of polysomy 17 with tumour stage and grade (figures represent number of tumours involved). Note the higher rates of polysomy 17 in the poorly differentiated and high stage tumours.

Table 3
HER2/neu gene amplification rates in several series

Method	Tumour number total (Ta, T1/T2)	% Amplified overall	% Amplified T2	Author, year, Ref.
Southern blotting	24(10/14)	4.1	7.1	Mellon and colleagues, 1996 [7]
Differential PCR	257 (225/22)	9	55	Underwood and colleagues, 1996 [12]
Quantitative PCR	57 (33/24)	32	59	Miyamoto and colleagues, 2000 [8]
FISH	135 (68/67)	7	13	Sauter and colleagues, 1993 [10]

PCR, polymerase chain reaction.
This table illustrates that several different methodologies have been used to assess *HER2/neu* gene amplification with reported rates varying from 4.1 to 32%. In every study, the rates are higher in the subset of pT2 tumours (7.1–59%).

Table 4
HER2/neu protein overexpression rates in several series

Antibody	Tumour number total (Ta, T1/T2)	Overexpression overall (%)	Overexpression in pT2 (%)	Author, year, Ref.
21N monoclonal	88 (52/36)	26	33	Sato and colleagues, 1992 [11]
Rabbit polyclonal	54 (9/45)	32	89	Moriyama and collagues, 1991 [9]
21N monoclonal	236 (178/56)	47	50	Underwood and colleagues, 1996 [12]
CB11 monoclonal	95 (53/42)	21	28	Mellon and colleagues, 1996 [7]

This table illustrates that several different antibodies have been used to assess HER2/neu protein overexpression with overall reported rates varying between 21 and 47%. However, the rates in pT2 tumours are higher in every study (28–89%).

of tumour polyploidy [2]. Taken together, these data suggest that alterations to genes on chromosome 17, or indeed duplication of the chromosome itself, is closely associated with the malignant progression of TCCs, suggesting this might play a role in the development of a malignant phenotype in TCC. The incidence of increased *HER2/neu* copy number parallels the incidence of polysomy 17.

Protein overexpression was assessed using IHC and the CB11 monoclonal antibody, and only membranous staining was assessed. Protein overexpression rates were also high, with 57% of tumours having either strong or moderate expression. The *HER2/neu* oncogene is a tyrosine kinase growth factor receptor and overexpression of the protein results in an increased cell proliferation rate and increased metastatic potential. This contributes to the malignant phenotype of the cell, and is consistent with the high rates of expression in poorly differentiated tumours, and the association with a poorer prognosis (Tables 3 and 4).

In contrast, the gene amplification rates were low (7%). This suggests that although the tumours have polysomy 17, mechanisms other than gene amplification account for the observed high protein overexpression rates. HER2/neu protein overexpression is believed to arise from a combination of two possible mechanisms. The first is gene amplification, which as this study demonstrates is uncommon in TCC of the bladder. The other mechanism is transcriptional activation that leads to a high number of each gene copy and this requires further research in TCC. For example, it has been observed that, in the absence of gene amplification, protein overexpression can occur due to the preferential binding of the *HER2/neu* transcription factor OB2-1 to cells that overexpress the protein [22]. Higher levels of such transcription factors, even in the absence of gene amplification result in increased HER2/neu protein overexpression. Stomach cancer cell lines, SNU-1 and SNU-16, have similar *HER2/neu* transcription rates with similar mRNA concentrations, but the SNU-l cells express the *HER2/neu* protein at a higher level than the SNU-16 cells. This is due to preferential translation of the mRNA from the SNU-1 cells [23].

Evidence from other cancers suggests that only tumours that are *HER2/neu* gene amplified respond to anti-*HER2/neu* therapy like trastuzumab [24]. Most breast cancers with strong protein overexpression ('3+') are also gene amplified and these respond well to anti-*HER2/neu* therapy. In this study, four of the tumours (5%, 4/75) would fit into this category. In breast cancer, if a tumour is scored as '2+' on IHC, then a response to anti-*HER2/neu* therapy is only seen if there is also gene amplification. None of the 15% (11/75) of tumours in this study scored as '2+' was gene amplified. Hence, if the data from breast cancer is extrapolated to high grade muscle-invasive TCC, then only 5% of patients would benefit from anti-*HER2/neu* therapy.

However, the high rates of polysomy 17, increased HER2 copy number and protein overexpression in these TCCs suggests that *HER2/neu* is important in the development of TCC. Further studies are needed to assess the potential mechanisms other than gene amplification that might be responsible for HER2/neu protein overexpression in TCC.

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