Cellular Oncology 32 (2010) 57–65
DOI 10.3233/CLO-2009-0497
IOS Press

HER2 expression in gastric cancer: Rare, heterogeneous and of no prognostic value – conclusions from 924 cases of two independent series

Heike Grabsch [a,*], Shivan Sivakumar [a], Sally Gray [b], Helmut E. Gabbert [c] and Wolfram Müller [d]

[a] *Pathology & Tumour Biology, Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK*
[b] *Department of Histopathology and Molecular Pathology, Leeds Teaching Hospitals NHS Trust, Leeds, UK*
[c] *Institute of Pathology, University of Düsseldorf, Düsseldorf, Germany*
[d] *Gemeinschaftspraxis Pathologie, Starnberg, Germany*

Abstract. *Background*: Patients with gastric cancer (GC) have a poor survival and biologicals such as Trastuzumab have not been used routinely in these patients. Existing data on HER2 expression and its clinical relevance in GC are still limited and controversial.

Methods: HER2 expression was investigated by immunohistochemistry in 418 GC from Germany and 506 GC from England. Results were compared to clinicopathological parameters and patient survival.

Results: Less than 10% of all GC showed HER2 expression in more than 5% of tumour cells and 91% of these were intestinal type GC. In both series, no relationship was found between HER2 expression, patient survival or TNM stage. Marked intratumoural heterogeneity was noted.

Conclusions: This is the largest study to date demonstrating in two independent series that HER2 expression is not related to gastric cancer patient prognosis and that only a very small subgroup of intestinal type GC may potentially respond to HER2 targeting therapy. Due to prominent intratumoural heterogeneity of HER2 expression in GC, HER2 testing in endoscopic biopsies before treatment will be prone to false negative results.

Keywords: Gastric cancer, HER2, prognosis, immunohistochemistry

1. Introduction

Although the incidence of gastric cancer (GC) has halved since the 1940s in Western countries, GC remains an important health issue and is the 4th commonest malignancy in the world [15]. In the West, more than 70% of patients are diagnosed at an advanced (unresectable) stage [24]. However, even with additional chemotherapy or chemoradiation, the median five year survival of patients with curatively resectable GC is still poor with 23% and 35%, respectively [3,29].

With the advent of "targeted therapies" it seems to be necessary to evaluate whether these new therapies could potentially be of benefit to GC patients by (i) establishing the frequency of expression of the potential target and (ii) investigating whether methods of predictive testing established for cancers of other organs are appropriate to be used in patients with GC.

Trastuzumab is a monoclonal antibody directed against the extracellular domain of the HER2 protein (also called: p185, HER2/neu, c-erbB2) and has demonstrated efficacy in GC cell lines [9,17] as well as xenograft models of GC [7,21]. Studies in primary breast cancer demonstrated that patients will only potentially respond to Trastuzumab treatment if tumour cells overexpress HER2 in the cell membrane and/or show *HER2* gene amplification [36]. So far, HER2 expression has been studied in relatively small series of

*Corresponding author: Dr. Heike Grabsch, Pathology and Tumour Biology, Leeds Institute of Molecular Medicine, Wellcome Trust Brenner Building, St. James's University Hospital, Beckett Street, Leeds LS9 7TF, UK. Tel.: +44 113 3438626; Fax: +44 113 3438431; E-mail: h.i.grabsch@leeds.ac.uk.

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GC patients showing controversial results regarding its prognostic value (for a recent review see Gravalos et al. [11]). Whilst the published frequency of HER2 expression in GC varies enormously between 8% [10, 31] and 91% of GC [1], there seems to be a consensus that HER2 expression is related to tumour morphology and is much more common in intestinal-type GC compared to diffuse-type GC [11]. However, the results published so far in primary GC including those proposing a new HER2 scoring system for GC [12] have neither been validated in a second independent series nor tested prospectively and thus, so far, no firm conclusions can be drawn from the published work.

Our study aims were (i) to assess whether the so called "DAKO-Score" which was originally developed for breast cancer is suitable for HER2 scoring in GC; (ii) to investigate the frequency and intratumoural distribution of membranous HER2 expression in two large series of primary GC from two different countries (total number of cases: $n = 924$); and (iii) to analyse the association of HER2 expression with clinicopathological parameters and patient survival.

2. Patients and methods

2.1. Gastric cancer series from Mainz, Germany (series A)

Patients (series A)

This patient cohort included 418 patients with sporadic gastric adenocarcinoma who had surgery at the University of Mainz, Germany, between 1.1.1980 and 31.12.1988. None of the patients received adjuvant or neoadjuvant chemotherapy, radiotherapy or both. The median age of the patients was 64.9 years, ranging from 23 to 90 years. Sixty percent of the patients ($n = 255$) were male. The median follow up time was 1.49 years (range: 0.09–9.09 years). At the end of the study period, 209 (50%) patients were still alive, 176 (42.1%) had died due to cancer, 18 (4.3%) had died due to other reasons and the cause of death was unknown for 15 (3.6%) patients. Patients who died within 30 days after surgery (postoperative mortality) were excluded from this study.

The following histopathological variables were available for analyses: morphological tumour type according to Laurén classification [18]; grade of tumour differentiation and morphological subtype according to WHO classification [33]; depth of tumour invasion (pT category) and lymph node status (pN category) according to TNM classification 3rd edition [13], and both, presence or absence of blood and lymphatic vessel invasion. Tissue for histological confirmation of distant metastases (pM category) was available in 23 patients and the surgical procedure in these patients was classified as "palliative resection". The study was performed according to the rules of the local ethics committee.

2.2. HER2 immunohistochemistry using full tissue sections (series A)

Four micron sections were cut from one representative tissue block from each patient containing GC including luminal tumour, tumour centre and invasion front as well as non neoplastic gastric mucosa. After dewaxing, rehydration and appropriate blocking procedures, sections were stained for HER2 using a monoclonal antibody (clone CB11, 1:20, Biogenex) and an avidin-biotin complex technique (Vector Elite Kit, 1:100). DAB (Dako) was used as chromogen according to the instructions of the manufacturer and tissue sections were counterstained with Mayer's haematoxylin, blued, dehydrated and coverslipped. Non neoplastic gastric mucosa was used as internal negative control. Incubation of the tissue sections with antibody diluent instead of primary antibody served as external negative control. Breast tissue known to be HER2 positive was used as external positive control.

2.3. Gastric cancer series from Leeds, England (series B)

Patients (series B)

This patient cohort included 506 patients with sporadic gastric adenocarcinoma who underwent surgical resection at the Academic Department of Surgery, Leeds General Infirmary, UK, between 1970 and 2004. None of the patients received adjuvant or neoadjuvant chemotherapy, radiotherapy or both. Surgery of ninety-six (19%) patients was classified as "palliative" due to incomplete tumour resection (R1) and/or histologically confirmed distant metastases (pM1). The median age of the patients was 71 years, ranging from 24 to 96 years. Sixty-two percent of the patients ($n = 312$) were male. The median follow-up time was 1.82 years (range: 0.09–20.56 years). Data from 47 (9%) patients who died within 30 days after surgery were excluded from survival analyses. At the end of the study period, 115 (23%) of patients were still alive, 220 (44%) had

died due to cancer, 156 (30%) had died due to other causes and 15 (3%) were lost from follow up.

The following histopathological parameters were available for analyses: depth of tumour invasion (pT) and lymph node status (pN) according to the TNM classification 5th edn [14]; grade of tumour differentiation according to the WHO classification [33] and morphological tumour type according to Laurén classification [18]. The study was performed with the approval of the Local Research Ethics committee.

2.4. HER2 immunohistochemistry using tissue microarray sections (series B)

Tissue microarrays (TMAs) were constructed as described previously by Simon et al. [27] using representative tissue blocks to sample three cores of 0.6 mm diameter randomly from GC with high tumour cell density (>50% tumour cells per area) and from non neoplastic gastric mucosa. Six cores were sampled from GC with low tumour cell density (<50% tumour cells per area). Each TMA contained also several different non gastric tissue samples (control tissues) to assess sensitivity and specificity of the immunohistochemistry procedure.

Immunohistochemistry (IHC) was performed on four micron sections of TMA blocks including an external negative control (omission of primary antibody), internal positive and negative controls (control tissues) and external positive controls (breast cancer tissue with known different HER2 expression levels) using the Dako Autostainer. After appropriate blocking procedures, anti-HER2 (clone CB11, 1:667, Novocastra) was used as primary antibody, Dako Envision Kit (Dako) as detection system and DAB (Dako) as chromogen according to the instructions of the manufacturer. Tissue sections were counterstained with Mayer's haematoxylin, blued, dehydrated and coverslipped.

2.5. Scoring of immunohistochemical staining

All sections were scored by an experienced histopathologist (WM) blinded to any of the clinicopathological parameters including patient outcome. Two parallel scoring systems were applied: (i) percentage of tumour cells with clearly visible membranous staining irrespective of the completeness or intensity of the membranous staining in individual cells; (ii) the "DAKO-Score", a method that was originally established for HER2 scoring in breast cancer and recently published as "ASCO recommendation" [32]: Score 0 (negative): no staining or any kind of membranous staining in less than 10% of tumour cells; Score 1 (negative): faint and incomplete membranous staining in more than 10% of tumour cells; Score 2 (equivocal): strong and complete membranous staining in more than 10% but less than 30% of tumour cells or any percentage of tumour cells with strong but incomplete membranous staining; Score 3 (positive): strong and complete membranous staining in more than 30% of tumour cells.

For statistical analyses, our gastric cancer HER2 scoring results were summarised into two categories: (i) "negative": less than 5% of tumour cells with any kind of membranous immunoreactivity or DAKO-Score 0 or 1; (ii) "positive": more than 5% of tumour cells with any kind of membranous immunoreactivity or DAKO-Score 2 or 3.

2.6. Statistical analyses

All statistical analyses were performed using SPSS 15.0 for Windows. Comparisons of the frequency of HER2 immunoreactivity for different groups were performed using the Wilcoxon–Mann–Whitney test (for two groups) or the Kruskal–Wallis test (for more than two groups). Different cut offs (5%, 10%, 20%, 30%, 40% and 50%) were tested. Agreement between the categories using the DAKO-Score and those established using a continuous percentage score with subsequent dichotomisation was tested using the Wilcoxon test for two related samples. The frequency of cases with a certain HER2 immunoreactivity score or percentage was compared between the different years in order to establish whether there is any relationship between HER2 staining pattern and age of paraffin blocks. Analyses of survival were performed using the Kaplan–Meier method [16] and differences between the patient groups were tested by the log rank test. Data from patients who died within 30 days after surgery (post-operative mortality) were excluded from survival analyses. Prognostic relevance was investigated by univariate and multivariate Cox regression analyses adjusting the multivariate model for known prognostic factors such as pT and pN. p-values less than 0.05 were considered significant.

3. Results

3.1. HER2 expression in non neoplastic gastric mucosa

The non-neoplastic gastric mucosa was negative in all cases (Fig. 1A, inset).

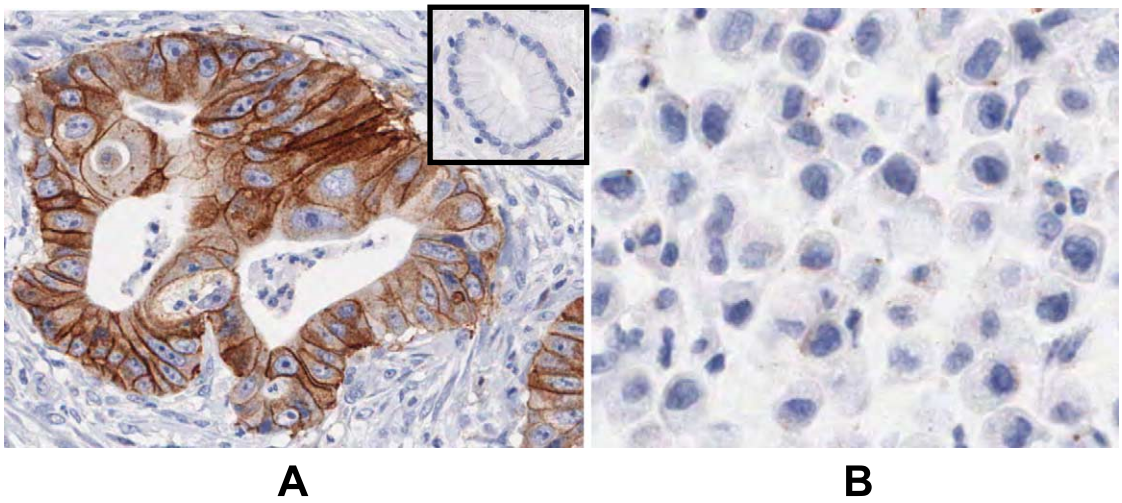

Fig. 1. HER2 expression in gastric cancer. (A) HER2 immunohistochemistry in intestinal-type gastric cancer demonstrates strong membranous HER2 staining in most tumour cells. Inset: normal gastric mucosa is HER2 negative. DAB (brown) and haemalaun (blue) counterstain, original magnification 63×. (B) HER2 immunohistochemistry in diffuse-type gastric cancer is negative. DAB (brown) and haemalaun (blue) counterstain, original magnification 63×. (The colors are visible in the online version of the article.)

3.2. HER2 expression in primary gastric cancer

Only cases where the cancer cells showed membranous staining were considered for scoring (Fig. 1). Prominent heterogeneity of HER2 staining was noted within most of the tumours in the full section IHC (series A) as well as between cores taken randomly from different locations within the same tumour (series B). This heterogeneity is reflected in the distribution of cases with HER2 positive tumour cells in the different percentage categories throughout all categories (Table 1).

In series A, 42 (10%) GC showed any HER2 expression. Thirty-six (9%) GC were classified as HER2 positive using a 5% cut off and 24 (6%) GC were HER2 positive using the DAKO-Score combining score 2 and 3 (Table 2). A good agreement (κ: 0.8; $p < 0.001$) was seen between the two different scoring systems with 406 (97%) GC being classified identically using either score and only 12 (3%) GC were classified "positive" using the 5% cut off but "negative" using the DAKO-Score.

The frequency of HER2 expression was significantly lower in series B ($p = 0.001$). In series B, 22 (4%) GC were classified as HER2 positive using the 5% cut off and 12 (2%) GC were HER2 positive using the DAKO-Score (Table 3). A good agreement (κ: 0.7; $p < 0.001$) was seen between the two different scoring systems with 496 (98%) GC being classified identically using either score and only 10 (2%) GC were classified "positive" using the 5% cut off but "negative" using the DAKO-Score.

3.3. Relationship of HER2 expression and histopathological parameters

The frequency of HER2 expression was significantly associated with GC morphology. With the exception of three GC in series A, only GC classified as intestinal-type by morphology were HER2 positive independent of the scoring system used (Tables 2 and 3). Statistically, the morphological tumour type according to WHO classification was significantly related to HER2 status in series A independent of the scoring system with most HER2 positive GC being classified as either tubular or papillary type (Table 2). The frequency of HER2 expression was significantly different between well differentiated GC and poorly differentiated GC in both series (Tables 2 and 3). No relationship was found between frequency of HER2 expression and depth of tumour invasion (pT category), lymph node status (pN category) or presence of lymphovascular invasion.

3.4. Relationship of HER2 expression and patient overall survival

In both series, depth of tumour invasion (pT) and nodal status (pN) were confirmed as independent prognostic marker in multivariate survival analyses (data

Table 1
Frequency of HER2 positive tumour cells/case comparing a continuous percentage score and the DAKO-Score

Series	Percentage of HER2 positive tumour cells[a]							DAKO-Score[b]			
	Total	0%	1–4%	5–25%	26–50%	51–75%	>75%	0	1	2	3
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
A	418 (100)	376 (90)	6 (1.4)	15 (3.6)	8 (1.9)	6 (1.4)	7 (1.7)	386 (92.3)	8 (2)	3 (0.7)	21 (5)
B	506 (100)	484 (95.7)	0 (0)	3 (0.6)	6 (1.2)	7 (1.4)	6 (1.2)	484 (95.7)	10 (2)	2 (0.3)	10 (2)

Notes: [a]Percentage of tumour cells with membranous staining irrespective of completeness or intensity.
[b]DAKO-Score 0 – staining in <10% of cells; 1 – incomplete membranous staining in >10% of cells; 2 – strong and complete membranous staining in more than 10% but less than 30% of tumour cells or any percentage of tumour cells with strong but incomplete membranous staining; 3 – strong and complete membranous staining in more than 30% of tumour cells.

Table 2
Clinical and pathological characteristics of patient series A by HER2 scoring method

	Total	HER2 DAKO-Score[a]			Percentage of HER2 positive tumour cells/case[b]		
	n	Negative	Positive	p-value	<5%	⩾5%	p-value
		n (%)	n (%)		n (%)	n (%)	
Palliative surgery							
No	411	377 (92)	24 (8)	0.299	365 (89)	36 (11)	0.197
Yes	17	17 (100)	0 (0)		17 (100)	0 (0)	
WHO classification							
Signet ring cells	112	112 (100)	0 (0)	**<0.001**	109 (97)	3 (3)	**0.017**
Papillary	38	31 (82)	7 (18)		31 (82)	7 (18)	
Tubular	163	150 (92)	13 (8)		144 (88)	19 (12)	
Mucinous	34	32 (94)	2 (6)		32 (94)	2 (6)	
Undifferentiated	71	69 (97)	2 (3)		66 (93)	5 (7)	
Laurén classification							
Intestinal-type	265	242 (91)	23 (9)	**0.003**	234 (88)	31 (12)	**0.011**
Diffuse-type	116	116 (100)	0 (0)		113 (97)	3 (3)	
Mixed-type	37	36 (97)	1 (3)		35 (95)	2 (5)	
Grade of differentiation							
G1/G2	112	99 (88)	13 (12)	**0.007**	97 (87)	15 (13)	0.105
G3	227	218 (96)	9 (4)		212 (93)	15 (7)	
G4	79	77 (97)	2 (3)		73 (92)	6 (8)	
Depth of tumour (pT)[c]							
pT1	96	93 (97)	3 (3)	0.391	91 (95)	5 (5)	0.4
pT2	188	174 (93)	14 (7)		170 (90)	18 (10)	
pT3/4	134	127 (95)	7 (5)		121 (90)	13 (10)	
Nodal stage (pN)[c]							
pN0	188	177 (94)	11 (6)	0.931	172 (91)	16 (9)	0.947
pN1	230	217 (94)	13 (6)		210 (91)	20 (9)	
Blood vessel invasion							
Negative	317	299 (94)	18 (6)	0.921	292 (92)	25 (8)	0.349
Positive	101	95 (94)	6 (6)		90 (89)	11 (11)	
Lymph vessel invasion							
Negative	219	210 (96)	9 (4)	0.133	205 (94)	14 (6)	0.09
Positive	199	184 (92)	15 (8)		177 (89)	22 (11)	

Notes: [a]DAKO-Score 0 or 1 summarised as "negative", DAKO-Score 2 and 3 summarised as "positive".
[b]Cases with <5% HER2 positive cells were considered "negative".
[c]TNM classification 3rd edn [13].

Table 3
Clinical and pathological characteristics of patient series B by HER2 scoring method

	Total n	HER2 DAKO-Score[a]			Percentage of HER2 positive tumour cells/case[b]		
		Negative n (%)	Positive n (%)	p-value	<5% n (%)	⩾5% n (%)	p-value
Palliative surgery							
No	410	399 (97)	11 (3)	0.342	391 (95)	19 (5)	0.514
Yes	96	95 (99)	1 (1)		93 (97)	3 (3)	
Laurén classification							
Intestinal-type	308	296 (96)	12 (4)	**0.008**	287 (93)	21 (7)	**0.001**
Diffuse-type	110	110 (100)	0 (0)		110 (100)	0 (0)	
Mixed-type	78	78 (100)	0 (0)		78 (100)	0 (0)	
Grade of differentiation							
G1	63	61 (97)	2 (3)	**0.024**	58 (92)	5 (8)	**<0.001**
G2	132	125 (95)	7 (5)		119 (90)	13 (10)	
G3	297	294 (99)	3 (1)		293 (99)	4 (1)	
Depth of tumour (pT)[c]							
pT1	66	66 (100)	0 (0)	0.45	65 (98)	1 (2)	0.411
pT2	176	170 (97)	6 (3)		167 (95)	9 (5)	
pT3	242	238 (98)	4 (2)		232 (96)	10 (4)	
pT4	19	17 (89)	2 (11)		17 (89)	2 (11)	
Nodal stage (pN)[c]							
pN0	167	162 (97)	5 (3)	0.258	160 (96)	7 (4)	0.538
pN1	209	203 (97)	6 (3)		197 (94)	12 (6)	
pN2	84	83 (99)	1 (1)		81 (96)	3 (4)	
pN3	38	38 (100)	0 (0)		38 (100)	0 (0)	

Notes: [a]DAKO-Score 0 or 1 summarised as "negative", DAKO-Score 2 and 3 summarised as "positive".
[b]Cases with <5% HER2 positive cells were considered "negative".
[c]TNM classification 5th edn [14]. Missing values: $n = 10$ (Laurén classification), $n = 14$ (grade), $n = 3$ (pT), $n = 8$ (pN).

not shown). In both series, univariate analyses showed that there is no statistically significant difference in overall survival in relationship to HER2 expression status comparing HER2 positive and HER2 negative cases irrespective of the scoring system (series A: $p = 0.074$ (5% cut off) and $p = 0.277$ (DAKO-Score); series B: $p = 0.347$ (5% cut off) and $p = 0.167$ (DAKO-Score; Fig. 2A and B). Interestingly, different trends are seen in the two studies: patients with DAKO-Score 2 or 3 tend to have a shorter survival in series A, but a longer survival in series B which is most likely related to the case mix, differences in mortality and follow-up time between the series (data not shown) as the combined survival analysis of both series brings both curves very close together (Fig. 2C).

No difference was found between the HER2 expression status comparing patients with palliatively and curatively resected GC in both series (Tables 2 and 3). HER2 expression status did not predict patient survival when the analysis was limited to palliative patients or to patients with intestinal-type GC.

3.5. *Relationship of HER2 staining pattern and year of surgery*

The frequency of cases classified as HER2 positive or HER2 negative using either the DAKO-Score combining score 2 and 3 or the 5% cut off was not significantly different over the different years of surgery ($p > 0.5$ series A and B). In series A, 50% of the patients had surgery between 1980 and 1984 and 58% of HER2 positive GC in series A were from the same time period evenly distributed over the years. In series B, 50% of the patients had surgery between 1970 and 1993 and exactly 50% of HER2 positive GC were detected between 1970 and 1993, all in different years except in 1987 where two positive cases were found in the same year.

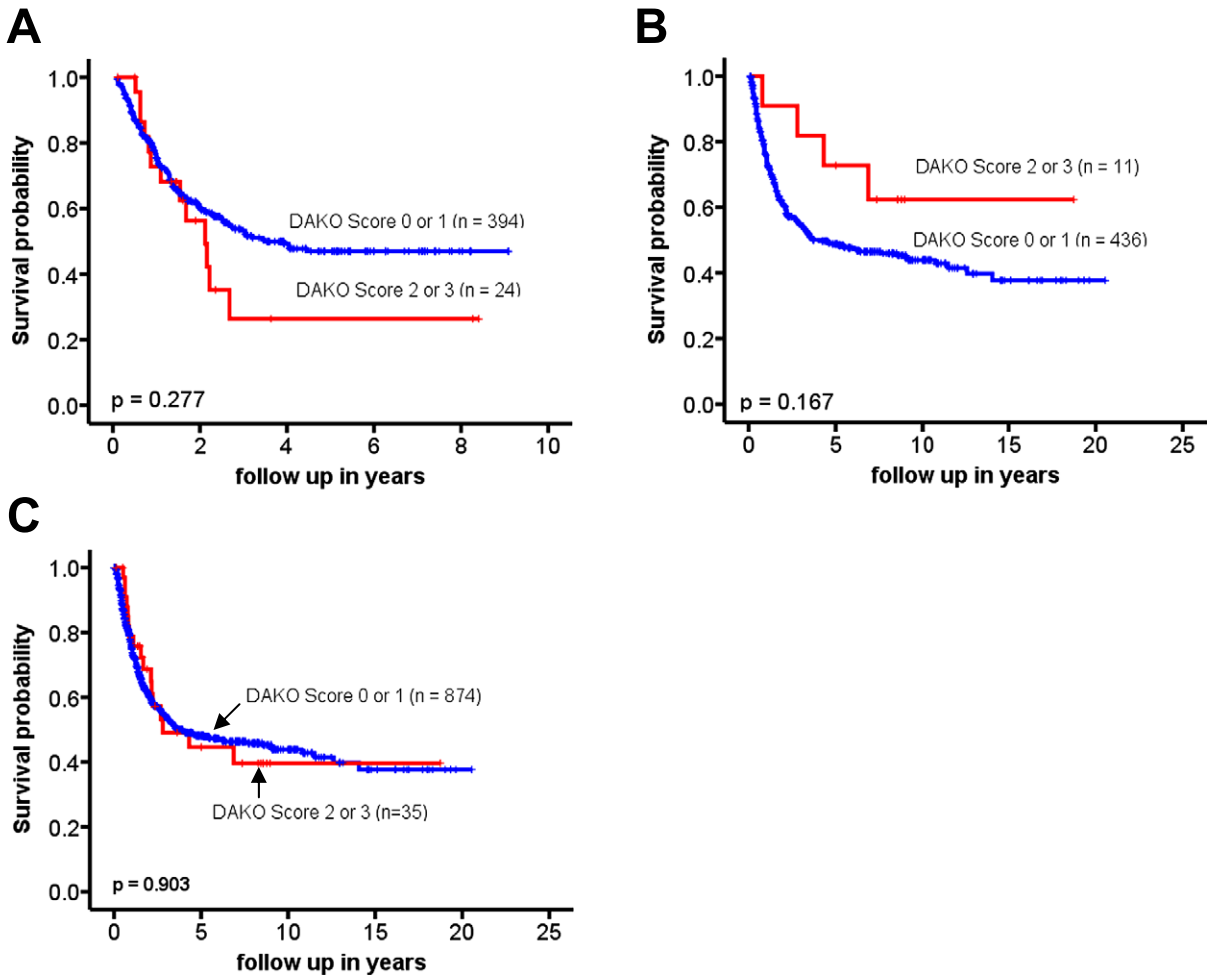

Fig. 2. Relationship between HER2 DAKO-Score and survival in gastric cancer. (A) Kaplan–Meier plot for series A comparing DAKO-Score 0 or 1 with DAKO-Score 2 or 3. (B) Kaplan–Meier plot for series B comparing DAKO-Score 0 or 1 with DAKO-Score 2 or 3. (C) Kaplan–Meier plot combining the data from both series and comparing DAKO-Score 0 or 1 with DAKO-Score 2 or 3.

4. Discussion

Patients with resectable gastric cancer have a poor prognosis even when treated with cytotoxic chemotherapy in combination with surgery [3–5]. The current therapeutic options for patients with GC appear to have reached a plateau of effectiveness and new treatment modalities including biological agents such as HER2 targeting agents need to be considered. Published studies investigating HER2 expression in GC have been very small, never been validated in a second independent set and therefore, results regarding the relationship of HER2 expression and clinicopathological data including patient survival are still controversial [11, 12].

The overall frequency of HER2 expression found in our two independent GC series is consistent with that of most other studies [2,10,20,25,35] but in contrast to Allgayer et al. [1] who demonstrated exceptionally high HER2 expression in 91% of GC. The comparison of the frequency of HER2 positive gastric cancer from patients who had surgery over a time period of more than 35 years demonstrated an even distribution of HER2 positive cases over the whole time period. This data suggests that HER2 can reliably be detected in paraffin blocks which are more than 30 years old. However, this may only be true if fresh sections are cut from such old blocks as done in the current study.

The significant difference in HER2 expression frequency between series A and B in the current study seems to be related to the prominent intratumoural heterogeneity of the HER2 staining pattern in GC observed in the full sections (series A) and therefore a

related sampling error when using randomly sampled tissue microarray cores (series B). This heterogeneity observed in GC has not been highlighted in previous publications and is in contrast to the homogenous HER2 staining and high concordance of results derived from full sections and tissue microarrays reported in breast cancer [6,37]. This raised the question whether the HER2 scoring system developed for breast cancer is actually applicable in GC or not. We therefore compared the HER2 breast scoring (so-called DAKO-score) with a continuous scoring scale. Our study in GC demonstrated a good agreement between HER2 scoring using a continuous scale and subsequent dichotomisation and the DAKO-Score for the classification of positive or negative cases. This indicates to us that a simple binary method, e.g. dichotomising cases at the 5% level irrespective of the amount of membranous staining is sufficient to classify GC into negative and positive HER2 cases.

The HER2 staining heterogeneity detected in our GC study implies that further studies are necessary to establish the concordance of the HER2 expression pattern between endoscopic gastric biopsies and full tissue sections before actively advocating HER2 immunohistochemistry in endoscopic biopsies to determine HER2 expression status before neoadjuvant treatment in GC. So far, from our study it seems unlikely that the results from biopsies will be concordant with those from full sections and other HER2 testing modalities such as determining the circulating HER2 level in the serum or using radionuclide molecular imaging of HER2 may possibly be better suitable to predict response to therapy in patients with GC.

Based on our study results, the proposed "consensus recommendation for HER2 scoring in gastric cancer" which separates into 4 different groups considering different forms of membranous staining and a 10% cut-off [12] seems to be unnecessary complicated, prone to higher interobserver variation and will result into too many potentially clinically meaningless subgroups in GC. However, we have to emphasise that our recommendation to use a 5% cut off score is only valid for the analyses of the relationship with clinicopathological data and patient survival. Due to the unavailability of suitable GC clinical material it is currently unknown what percentage of tumour cells need to express HER2 in which pattern in order for a gastric cancer to respond to HER2 targeting therapy as, for example, Trastuzumab treatment effects have so far only been tested using GC cell lines known to overexpress HER2 in all cells [7,21].

The current study confirmed in two independent large population-based series that HER2 expression status is not a prognostic factor in patients with GC which is in agreement with some authors [2,26,28] but in contrast to other studies [8,22,23,30]. The disagreement is probably related to smaller sample size, different case mix, use of different primary antibodies, detection systems and scoring methods in previous studies. Our study also confirmed that the frequency of HER2 expression is related to the morphological tumour type and is much higher in intestinal-type and well-differentiated GC compared to diffuse-type GC [2,19,34] providing further evidence for the hypothesis that intestinal and diffuse-type GC develop along different molecular pathways. However, as only a small fraction of intestinal-type GC are HER2 positive this indicates very clearly that even GC with identical morphological phenotype have a different molecular phenotype and will most likely require different treatment strategies.

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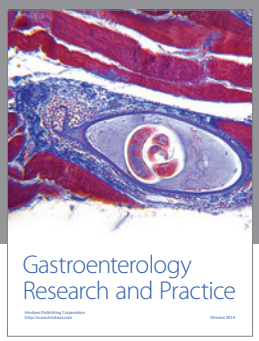

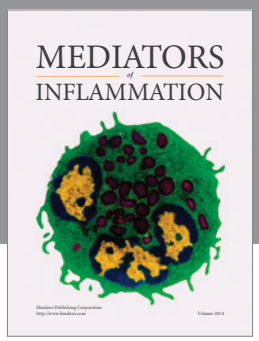

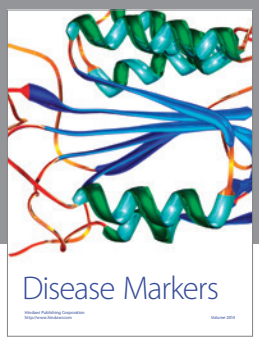

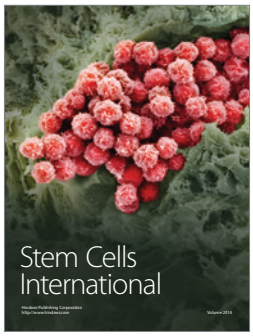

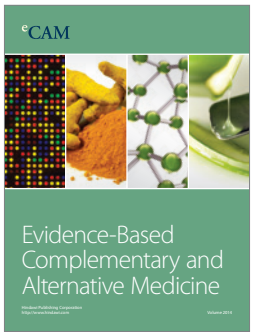

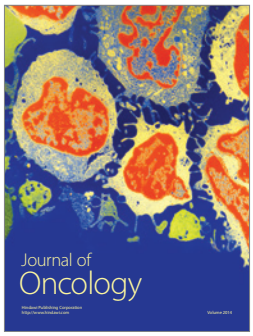

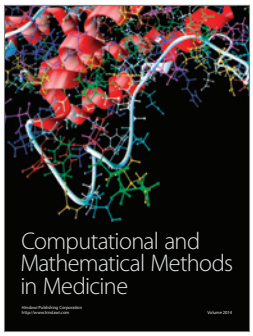

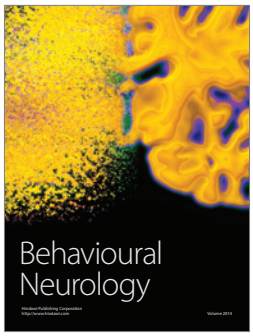

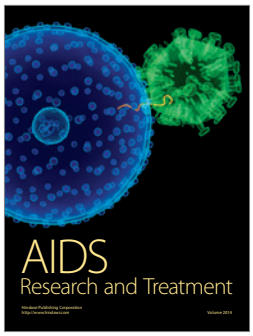

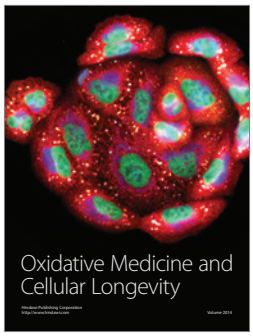

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