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RESEARCH

HER2 in Gastric Cancer: An Immunohistochemical Study on Tissue Microarrays and the Coressponding Whole-Tissue Sections with a Supplemental Fish Study

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Abstract Since focal HER2 expression is an issue in GC, TMA construction from the paraffin-embedded surgically-obtained tissue may not reflect its real status. The aim of this study was to assess the HER2 status in tissue microarrays (TMAs) and the corresponding whole sections using HercepTest immunohistochemistry (IHC), and to correlate it and to assess the concordance of HER2 IHC and fluorescence in situ hybridization (FISH) in TMAs. Concordance of the HER2 expression status for 302 cases of gastric cancer using 9 paired TMAs was evaluated using a 2-mm core size and 305 corresponding whole sections. Concordance of the IHC and FISH HER2 status was compared. In addition,, the HER2 status was compared to clinicopathological characteristics and patients' survival. Using the whole-section approach, HER2 over-expression was found in 25.2 % (HER2 3+ 6.6 %, HER2 2+ 18.7 %) of tumours. The overall concordance of IHC between the cores and the whole section was 84.9 %; 15.1 % of the tumours showed HER2 amplification. The overall concordance of IHC and FISH on cores was 75.7 %. The level of amplification correlated with the IHC score. Relationship between the intestinal and papillary types and tumour grade was observed for tumours with over-expression and amplification, whereas tumour location was related only to over-expression. There was a statistically significant difference in the overall survival of the patients, which was related to HER2 amplification. In conclusion, good concordance of the IHC HER2 results between tissue cores in TMA and whole sections, and excellent concordance of the IHC and FISH results on tissue cores was found. At least a part of the observed IHC HER2 heterogeneity could very likely be explained by fixation artifacts. With adequate fixation, a higher concordance of IHC HER2 between the cores and the whole sections can be expected. The TMA approach could enable an easier analysis of more than one representative tumour block.

Keywords FISH · Gastric carcinoma · HER2 · Immunohistochemistry · TMA · Whole sections

Abbreviations

CISH	Chromogenic in situ hybridization
EMA	European Medicine Agency
FISH	Fluorescent in situ hybridization
GC	Gastric carcinoma
HER2	Human epidermal growth factor receptor 2
HGA	High grade amplification
IHC	Immunohistochemistry
LGA	Low grade amplification
NPV	Negative predictive value
PPV	Positive predictive value
SISH	Silver in situ hybridization

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TMA Tissue microarray
ToGA Trastuzumab for gastric cancer

Introduction

Despite the fact that the incidence of gastric cancer (GC) has declined significantly in the last 50 years, it remains one of the most common cancers worldwide, with more than one million new cases diagnosed each year; it represents the fourth most common human cancer [1]. Moreover, the mortality rate from GC is constantly high in almost every part of the world, with overall 5-year relative survival rates approximate to 20 % [2, 3], except in Japan where mass screening programmes, staging systems and treatment ensure a 5-year survival rate of approximately 60 % [4]. Numerous randomised studies investigating the therapies for advanced GC have been conducted throughout the world, achieving some survival prolongation, but without establishing a globally-accepted standard chemotherapy regimen [5]. A better understanding of the molecular basis of cancer has contributed to the development of molecular-targeted therapies. One of the targets is the HER2 protein, a 185-kd transmembrane tyrosine-kinase receptor, encoded by a HER2 gene, located on the chromosome 17 (17q12-q21). In carcinomas, HER2 acts as an oncogene and recent studies indicate its role in the development of numerous types of human cancer, such as ovarian, endometrial, salivary gland, lung, oesophageal and gastric cancer [6, 7]; its role and clinical significance are best established in breast carcinomas [8].

Over-expression of the HER2 protein in GC has been demonstrated in a large number of studies [9, 10], with large discrepancies in the reported HER2-positivity rates ranging from 8.1 % to even 91 % [9–12]. The explanation of this is probably complex and includes different demographic and socio-economic circumstances; however, the most important factors are the use of different, non-standardised assays, different scoring criteria and subjectivity of pathologists' interpretation. The most recent studies demonstrated HER2-positivity rate to be 15–25 % [11, 13–16]. Furthermore, HER2 positivity in GC was reported to be associated with adverse prognosis, and a more aggressive disease [12, 14, 17].

The technology of TMAs already showed its applicability in the assessment of HER2 over-expression and amplification in breast carcinoma [18]. Since focal HER2 expression is an issue in GC [11, 16, 19], TMA construction from the paraffin-embedded surgically-obtained tissue may not reflect its real status. In view of this problem, we conducted a comparative study with the following aims:

- To evaluate the frequency of HER2 over-expression and amplification in GC in our series of patients, and to correlate it with clinicopathological parameters.
- To evaluate the concordance of HER2 over-expression between whole-tissue sections and the corresponding tissue cores using the HercepTest and new guidelines for HER2 scoring in GC.
- To evaluate the concordance of the immunohistochemistry and FISH results for tissue cores.

Materials and Methods

Patients

Three hundred two from a total of 989 patients with early and advanced GC (cardia and non-cardia cancers) who had undergone surgery between 2000 and 2008 were identified from the files of the Department of Pathology, Institute of Oncology, Ljubljana, Slovenia. Routinely fixed (over-night in a 10 % buffered formalin), paraffin-embedded tumour samples were used in this study. Hematoxylin-eosin-stained slides and the respective paraffin blocks were retrieved from the archives and the tumours were histologically re-classified by one of the authors (GG) according to the Lauren and WHO criteria. Patients were selected by chance; the only criterion for inclusion in the study was a sufficient number of tumour paraffin blocks per surgical sample to be able to ensure enough material for whole-section cutting and construction of TMA. At least 2 representative tumour blocks were present for every single patient. None of the patients received pre-operative chemotherapy, radiotherapy or both. Patients and lesion characteristics are shown in Table 1. 199 males (65.9 %), and 103 females (34.1 %) were included in the study. The age of the patients ranged from 33 years to 87 years (67±12 in average). Follow up was available for 290 patients; the median follow-up time was 2.28 years (range: 0.04–10.27). By the end of this study (December 2010), 201 (67 %) patients died; 156 (76.4 %) due to cancer, 22 (10.8 %) of other reasons and the cause of death was not known for 28 of them (13.8 %). 101 patients (33 %) were still alive. In a statistical analysis of survival curves for the amplification status, 4 patients with HER2-amplified GC (2 with HGA and 2 with LGA) were excluded from the analysis because surgical resection had been performed at an early stage of the disease (pT1), influencing its natural course.

Clinical data were collected in a blinded manner from the patients' documentation and Cancer Registry. The following histopathological variables were studied: tumour size, morphological tumour type according to the Lauren classification, morphological sub-type and tumour grade according to the WHO classification, depth of tumour invasion (pT category), lymph node status (pN category), presence or absence of vascular, lymphatic and perineural invasion and, for 159 patients, presence or absence of distant metastases. The study was preformed according to the rules of the National Ethics Committee and Declaration of Helsinki.

Table 1 Patients and lesion characteristics and comparison of clinicopathologic findings between HER2-negative and HER2-positive gastric carcinomas evaluated by IHC

	Total n, (%)	HER2 over-expression		*P* value
		Negative (0, 1+)	Positive (2+, 3+)	
	n=302	225	77	
Location				
Gastric ca	244 (81)	188 (77)	56 (23)	0.037
ca of GEJ	58 (19)	37 (64)	21 (36)	
Gender				
Male	199 (66)	150 (75)	49 (25)	0.628
Female	103 (34)	75 (73)	28 (27)	
Lauren classification				
Intestinal	184 (61)	122 (66)	62 (34)	<0.0001
Diffuse	52 (17)	50 (96)	2 (4)	
Mixed and others	66 (22)	53 (80)	13 (20)	
WHO classification				
Tubular	154 (51)	114 (74)	40 (26)	<0.0001
Papillar,tubulopapillar	27 (9)	7 (26)	20 (74)	
Mucinous	16 (5)	12 (75)	4 (25)	
Signet ring cell	100 (33)	87 (87)	13 (13)	
Other	5 (2)	5 (100)	0 (0)	
Grade of differentiation				
G1/G2	94 (31)	49 (52)	45 (48)	
G3	93 (31)	76 (82)	17 (18)	
G4	115 (38)	100 (87)	15 (13)	<0.0001
Tumour size				
<5 cm	125 (41)	92 (74)	33 (26)	0.762
>5 cm	177 (59)	133 (75)	44 (25)	
Depth of infiltration				
pT1	31 (10)	21 (68)	10 (32)	0.166
pT2	133 (44)	93 (70)	40 (30)	
pT3	130 (43)	104 (80)	26 (20)	
pT4	8 (3)	7 (88)	1 (12)	
Nodal stage				
pN0	79 (26)	56 (71)	23 (29)	0.514
pN1	95 (32)	75 (79)	20 (21)	
pN2	79 (26)	60 (76)	19 (24)	
pN3	49 (16)	34 (69)	15 (31)	
Vascular invasion				
Positive	57 (19)	42 (74)	15 (26)	0.982
Negative	183 (61)	137 (75)	46 (25)	
Unknown	62 (20)	46 (74)	16 (26)	
Lymphatic invasion				
Positive	127 (42)	97 (76)	30 (24)	0.227
Negative	6 (2)	6 (100)	0 (0)	
Unknown	169 (56)	122 (72)	47 (28)	
Perineural invasion				
Positive	175 (58)	86 (80)	22 (20)	0.295
Negative	108 (36)	126 (72)	49 (28)	
Unknown	19 (6)	13 (68)	6 (32)	
Metastatic disease				
Positive	78 (26)	60 (77)	18 (23)	0.604
Negative	82 (27)	63 (77)	19 (23)	
Unknown	142 (47)	102 (72)	40 (28)	

Whole-Section Cutting and TMA Construction

After reviewing all H&E tumour slides, tumours were re-classified according to the current TNM and Lauren classification. A representative slide and the corresponding tumour block were selected for each patient. On the representative slide that included the area of invasive GC, two areas of invasive carcinoma with high tumour cell density were marked by a pathologist (GG). From the corresponding paraffin block, five 3-μm-thick sections were cut for each sample. After that, TMAs were constructed by sampling two cores of a 2-mm diameter for carcinomas that were classified as intestinal, diffuse or mixed with intermingled components. For mixed-type carcinomas with separated components, 4 cores of a 2-mm diameter were sampled (2 from each tumour component). Cores were arranged as a 40-core format array into a new paraffin block. Each TMA contained also the non-gastric (liver) tissue and the tissue of breast carcinoma with an over-expressed and amplified HER2. TMAs were constructed with the MTA-1 manual tissue arrayer (Beecher Instruments Inc., Silver Spring, MD, USA). In 3 mixed GC, the intestinal and diffuse components were not present in the same paraffin block therefore two whole sections had to be cut. Altogether, 305 whole sections were cut, and 668 tissue cores were punched out and arranged into 9 paired TMAs.

Immunohistochemical Staining and Scoring

Immunohistochemistry was performed on a 3-μm whole-tumour sections and a 3-μm section of TMA blocks including an internal positive (breast cancer) and negative (liver) controls and external controls (DAKO cell lines). The HercepTest ™ was used according to the manufacturer's protocol.

For the interpretation of the results for whole sections and cores, a 4-step scale (0, 1+, 2+ and 3+) according to the Consensus Panel recommendations on HER2 scoring for GC [11] was used with additional Rüschoff's criteria [16]. The following criteria were used for scoring: 0, no reactivity or membranous reactivity in less than 10 % of cells; 1+,

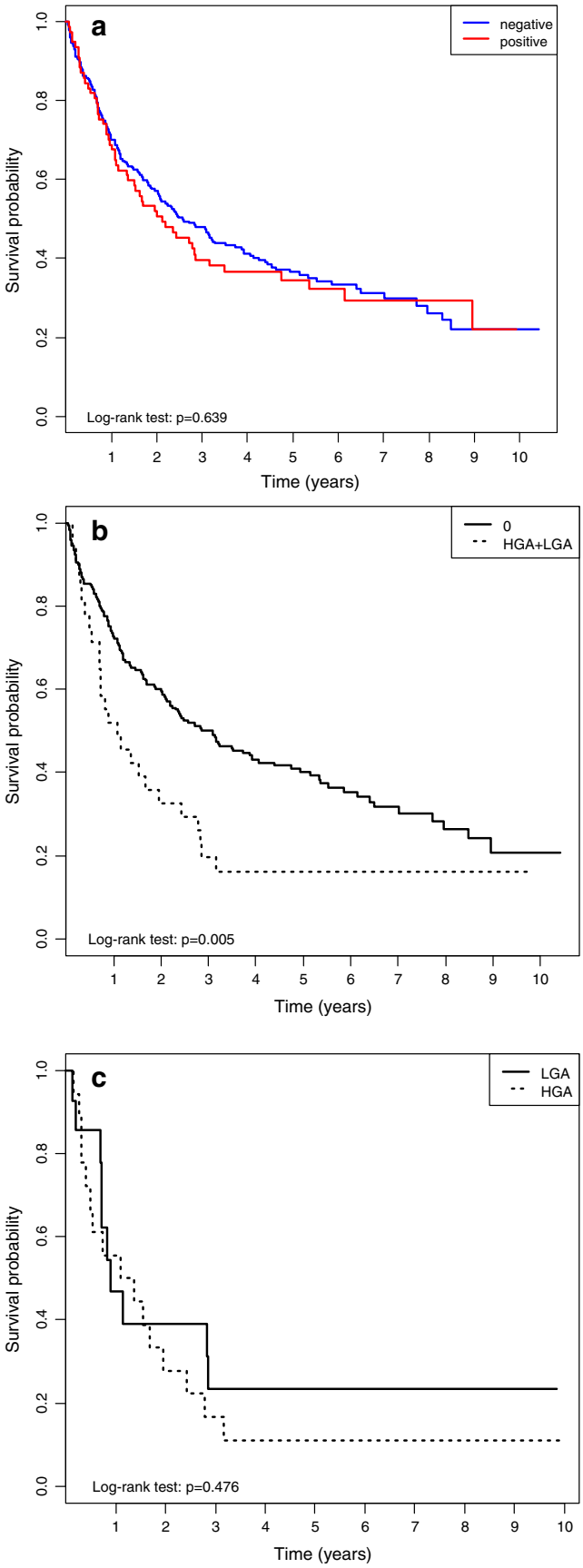

Fig. 1 **a** Kaplan-Meier plot of the survival probability for gastric cancer patients according to HER2 over-expression. **b** Kaplan-Meier plot of the survival probability for gastric cancer patients according to the HER2 amplification status. **c** Kaplan-Meier plot of the survival probability for gastric cancer patients according to the HER2 amplification level

faint/barely perceptible membranous reactivity in 10 % of cells or higher; 2+, weak to moderate complete or basolateral membranous reactivity in 10 % of tumour cells and higher; 3+, strong complete or basolateral membranous reactivity in 10 % of tumour cells and higher. As the TMA cores were tested analogous to biopsies, the 10 % cut-off was not regarded as the final scoring used in whole sections. Cases with scores of 2+ and 3+ were considered positive for HER2 over-expression. The HER2 status of the whole slides was compared to the HER2 status of the tissue cores. For those whole sections that were represented with two cores (cases of mixed-type carcinomas with separated diffuse and intestinal components), the core with a higher HER2 score was considered the final score.

FISH Staining and Scoring

Technically, the FISH procedure for the assessment of HER2 amplification was performed as described previously [18] using a FDA-approved PathVysion HER2 DNA Probe Kit and the Paraffin Pretreatment Kit (both Abbott-Vysis, Inc, Downers Grove, IL, USA). FISH was performed on all TMAs and on 7 whole sections with HER2 3+ foci <10 % that were not represented in the cores. The HER2 gene and centromere 17 signals were analysed thoroughly in all cores' areas under the fluorescent microscope, and were finally counted in at least 20 nuclei. If the ratio between HER2 and centromere 17 signals was ≥2, HER2 was considered amplified, in the range of 2–3 as low-grade and >3 as high-grade amplification. All amplified and ambiguous cases, as well as all cases scoring IHC 2+, were re-checked by one of the co-authors (CJA) using the Ariol® automated system. FISH scoring was performed blinded to the IHC results.

Statistical Analysis

The differences between the HER2-positive and HER2-negative groups were tested using the chi-square test or the Fischer's exact test, when appropriate. Survival curves were calculated by the Kaplan-Meier method and compared using the log-rank test. Cohen's kappa coefficient was used to measure the agreement between whole sections and tissue cores. The sensitivity, specificity and the overall concordance rate were also calculated. Statistical packages R 2.11.1 and PASW 18 were used to perform the analyses.

Table 2 Concordance between the results of HER2 IHC in tissue core 1 and whole sections

Core 1 *n*, (%)	Whole section *n*, (%)		Total *n*, (%)	NPV (%)	PPV (%)
	IHC HER2 negative (0, 1)	IHC HER2 positive (2, 3)			
IHC HER2 negative (0, 1)	183 (65)	25 (9)	208 (74)	88.0	
IHC HER2 positive(2, 3)	18 (6)	57 (20)	75 (26)		76.0
Total	201 (71)	82 (29)	283		

Cores were uninformative for 19 whole sections

Results

Immunohistochemistry

HER2 Expression on Whole-Tissue Sections, its Relation to Clinicopathological Parameters and Patient Overall Survival

IHC was technically successful in all whole-tissue sections. A total of 20 sections were evaluated as 3+ cases (6.6 %), 57 as 2+ (18.7 %), 38 as 1+ (12.5 %), and 190 as 0 cases (62.2 %). From 3+ cases, 14 (70.0 %) were well- to moderately-differentiated tubulopapillary carcinomas, 3 (15 %) were well- to moderately-differentiated tubular carcinomas, 2 (10 %) were grade III tubular carcinomas, and 1 (5 %) was the intestinal component of a mixed carcinoma with separated intestinal and diffuse components. All of them were strongly and homogeneously positive. There were 9 cases (2.98 %) that were scored as negative but showed foci of HER2 3+ in <10 % of the tumour. HER2 over-expression was significantly associated with tumour location, histological tumour type (Lauren and WHO classifications) and grade of differentiation. The results and their relation to clinicopathological parameters are shown in Table 1. The median survival time for IHC-negative cases (Fig. 1-a) was somewhat longer (31 months vs. 25.3 months; CI of 24.6–44.6 and CI of 16.5–41.8), but the difference was not statistically significant (p=0.639).

Concordance Between the Results for HER2 Over-Expression Determined by IHC on Tissue Cores and the Corresponding Whole-Tissue Sections

Of all 668 punched-out cores, a total of 36 (5.4 %) could not be assessed with IHC due to technical difficulties or uninformative cores.

For the first core (results shown in Table 2), the overall concordance rate was 84.8 %(κ=0.62, 95 % CI: 0.51–0.72). Sensitivity was 69.5 % (58.4–79.2 %), and specificity was 91.0 % (95 % CI: 86.2–94.6 %). The NPV of a negative IHC result in the core was 88.0 %; the PPV of a positive result in the core was 76.0 %.

For the second core (results shown in Table 3), the overall concordance rate was 86.3 % (κ=0.67, 0.58–0.76). Sensitivity was 67.4 % (57.0–76.6), and specificity was 95.8 % (91.9–98.1). The NPV of a negative IHC result in the core was 85.4 %, while the PPV of a positive result in the core was 88.9 %.

If whole sections and tissue cores are compared, taking into account the core with the highest HER2 score, the following results are obtained: the overall concordance rate is 83.5 % (κ=0.63, 0.53–0.72), sensitivity is 93.4 % (85.3–97.8 %), and specificity is 80.1 % (74.2–85.1 %). The NPV of a negative IHC result in the core is 97.3 %, and the PPV of

Table 3 Concordance between the results of HER2 IHC in tissue core 2 and whole sections

Core 2 *n*, (%)	Whole section *n*, (%)		Total *n*, (%)	NPV (%)	PPV (%)
	IHC HER2 negative (0, 1)	IHC HER2 positive (2, 3)			
IHC HER2 negative (0, 1)	182 (64)	31 (11)	213 (75)	85.4	
IHC HER2 positive (2, 3)	8 (3)	64 (22)	72 (25)		88.9
Total	190 (67)	95 (33)	285		

Cores were uninformative for 17 whole sections

a positive result in the core is 61.7 %. The results are shown in Table 4.

Fish

Concordance Between the Immunohistochemical and FISH Results for Cores

The HER2 status could be determined by FISH in 505 cores (75.6 %); in the remaining 163 cores (24.4 %), this was not possible due to the inadequate quality of reaction manifested as a lack of or weak signals, strong background fluorescence or washed-out cores. Furthermore, 6 cores with evaluable FISH results could not be compared with the IHC cores, as the IHC cores were washed out, and the IHC results were not available. Thus, HER2 over-expression and amplification could be compared in 499 (74.7 %) cores. HER2 gene amplification was demonstrated in 69 cores (13.8 %). Among them (Table 5), all IHC 3+ cores (41/41) showed HER2 amplification, which was also the case in 11.5 % of IHC 2+ (14/122) cases, in 9.7 % of IHC 1+ (10/103) cases and in 1.7 % of IHC 0 (4/233) cases. The overall concordance between IHC and FISH was 75.5 %.

In HER2-amplified cores, the level of amplification correlated with the IHC score. Whereas all cores with the IHC score of 0 or 1(14/14) and a majority (11/14) of cores with IHC 2+ showed LGA with ratios of 2–3, the proportion of such cases in the amplified cores with the IHC score of 3 was 7.3 % (3/41), respectively. Therefore, a vast majority of IHC 3+ cores (92.7 %) had HGA. Results are shown in Table 5.

Comparison of the FISH Results in Core Pairs

A total of 105 (31.4 %) of the 334 core pairs could not be compared due to at least one uninformative core. From 229 informative core pairs (68.6 %), both cores were non-amplified in 194 of them (84.7 %). In 19 (8.3 %) and 13 (5.7 %) pairs, both cores showed HGA or LGA, respectively, in the entire core area. In 3 core pairs (1.3 %), the discrepancy between the cores was found, with no amplification in one core and with foci of HGA in the second core. A comparison of discrepant cases with whole-section IHC showed three cases where HER2 3+ areas represented 10–20 % of an IHC-overall negative tumour.

Table 5 Concordance between the results of HER2 IHC and FISH in tissue cores

	IHC 0	IHC 1+	IHC 2+	IHC 3+	Total
FISH +					
LGA	4	10	11	3	28
HGA	0	0	3	38	41
FISH -	229	93	108	0	430
Total	233	103	122	41	499

FISH Results for Whole-Tissue Sections

In 7 cases with IHC 3+ <10 % of the tumour that were not displayed in TMAs, FISH was performed on whole sections. In all 7 cases, FISH showed amplification, mainly HGA, in IHC HER2 3+ foci in otherwise non-amplified cancer.

HER2 Amplification and its Correlation to Clinicopathological Parameters and Overall Survival

The comparison of clinicopathological findings regarding the HER2 amplification status and the differences with regard to LGA and HGA are shown in Tables 6 and 7, respectively. HER2 amplification was significantly associated with histological tumour type (Lauren and WHO classifications) and the grade of differentiation. As for the level of amplification, significant differences were found for tumour of a histological type according to the WHO classification and the grade of differentiation. The median survival time (Fig. 1b) was shorter for amplified cases (13.1 months vs. 37.1 months; CI of 8.6–26.4 and 33.4–48.6), and the difference was statistically significant (p=0.00531). Cases with LGA and HGA also showed a difference in the median survival time (Fig. 1c) (LGA:HGA=10.6:14.8; CI of 8.57–6.00 and NA-33.4), but without statistical significance (p=0.476).

Discussion

Since focal HER2 expression is an issue in GC [11, 16, 19, 20], suitability of TMAs for the assessment of the HER2

Table 4 Concordance between the results of HER2 IHC in tissue core with the highest HER2 score and whole sections

Core with highest HER2 score *n*, (%)	Whole section *n*, (%)		Total *n*, (%)	NPV (%)	PPV (%)
	IHC HER2 negative (0, 1)	IHC HER2 positive (2, 3)			
IHC HER2 negative (0, 1)	177 (59)	5 (2)	182 (61)	97.3	
IHC HER2 positive (2, 3)	44 (15)	71 (24)	115 (39)		61.7
Total	221 (74)	76 (26)	297		

Both cores were uninformative for 5 whole sections

Table 6 Comparison of clinicopathologic findings between HER2-amplified and HER2-non-amplified gastric carcinomas evaluated by FISH

N=237	HER2 amplification		*P* value
	Negative 201 (85)	Positive 36 (15)	
Location			
Gastric ca	163 (85)	28 (15)	0.643
ca of GEJ	38 (83)	8 (17)	
Gender			
Male	133 (86)	21 (14)	
Female	68 (82)	15 (18)	0.364
Lauren classification			
Intestinal	114 (79)	31 (21)	0.003
Diffuse	37 (97)	1 (3)	
Mixed and others	50 (93)	4 (7)	
WHO classification			
Tubular	103 (88)	14 (12)	<0.0001
Papillar, tubulopapillar	8 (32)	17 (68)	
Mucinous	13 (100)	0 (0)	
Signet ring cell	74 (95)	4 (5)	
Other	3 (75)	1 (25)	
Grade of differentiation			
G1/G2	52 (68)	25 (32)	<0.0001
G3	65 (92)	6 (8)	
G4	84 (94)	5 (6)	
Tumour size			
<5 cm	83 (87)	12 (13)	0.369
>5 cm	118 (83)	24 (17)	
Depth of infiltration			
pT1	18 (82)	4 (18)	0.755
pT2	89 (84)	17 (16)	
pT3	50 (85)	15 (15)	
pT4	6 (100)	0 (0)	
Nodal stage			
pN0	59 (92)	5 (8)	0.095
pN1	64 (84)	12 (16)	
pN2	88 (85)	9 (15)	
pN3	28 (74)	10 (26)	
Vascular invasion			
Positive	41 (87)	6 (13)	0.634
Negative	117 (83)	24 (17)	
Unknown	43 (88)	6 (12)	
Lymphatic invasion			
Positive	89 (86)	14 (14)	0.653
Negative	3 (100)	0 (0)	
Unknown	109 (83)	22 (17)	
Perineural invasion			
Positive	116 (84)	23 (16)	0.766
Negative	74 (87)	11 (13)	
Unknown	11 (85)	2 (15)	
Metastatic disease			
Positive	50 (82)	11 (18)	0.230
Negative	62 (91)	6 (9)	
Unknown	89 (82)	19 (18)	

status may be questioned. To the best of our knowledge, there is no study that analysed the HER2 status of GC in a TMA setting and on the corresponding whole-tissue sections, using either IHC or FISH. The only similar study was reported by Grabsch's group [19]; however, in that study, HER2 over-expression in TMAs and whole sections was compared between two different patients' cohorts, thus on non-corresponding tissues, while in a recent study published by Kunz et al. [21], only IHC- and FISH-discrepant cases were analysed for HER2 IHC expression in full cross sections.

The issue of the heterogeneity of HER2 in GC is still not well defined. There is no formal definition for it, and it is not clear whether it should be based on morphological, immunohistochemical or ISH criteria. While in some studies, a ≤10 % cut-off of IHC HER2 3+ foci is used as a synonym for heterogeneity [11, 21], reporting the latter to be in the range of 5 %, some other publications quantified the proportion of immunoreactive glands [11, 20] and amplified glands[20], reporting a heterogeneity rate of 50 %. In our study, scoring of IHC HER2 for GC was performed in accordance with the accepted criteria [11, 16]. In whole-section scoring, a 10 % cut-off was considered for scoring, while TMA cores were tested analogous to biopsies with a cluster of at least 5 immunoreactive cells. Regarding whole-section immunohistochemistry, heterogeneity was considered in cases where there was an obviously different, IHC 3+ clone representing <10 % of the tumor area (Fig. 2); in all other cases, we found an "uneven HER2 reaction", to use a more appropriate term. Nine tumours (2.98 %) were designated as heterogeneous tumours in which <10 % HER2 3+ foci was noticed, and that is in the range of some recent reports [11, 21]. In many cases, an uneven immunohistochemical reaction (Fig. 3) with some intermingled 1+ and 2+ areas, sometimes even small 3+ areas, was present, not only between tumour areas with similar morphology but also in the same tumour gland. In at least 5 of those cases, we have noticed a more intensive immunohistochemical reaction on the luminal and serosal surface of the tumour, whereas the central part of the tumour showed a weaker reaction or was not reactive at all (Fig. 4). The most probable explanation for that finding is false IHC negativity due to fixation artifacts.

Table 7 Comparison of clinicopathologic findings between HER2-amplified gastric carcinomas with HGA and HER2-amplified gastric carcinomas with LGA evaluated by FISH

N=36	HER2 amplification		*P* value
	HGA (ratio>3) 20 (56)	LGA (ratio2-3) 16 (44)	
Location			
Gastric ca	15 (54)	13 (46)	0.709
ca of GEJ	5 (63)	3 (37)	
Gender			
Male	13 (62)	8 (38)	0.500
Female	7 (47)	8 (53)	
Lauren classification			
Intestinal	19 (61)	12 (39)	0.190
Diffuse	0 (0)	1 (100)	
Mixed and others	1 (25)	3 (75)	
WHO classification			
Tubular	5 (36)	9 (64)	0.008
Papillar, tubulopapillar	14 (82)	3 (18)	
Mucinous	0 (0)	0 (0)	
Signet ring cell	1 (25)	3 (75)	
Other	0 (0)	1 (100)	
Grade of differentiation			
G1/G2	18 (72)	7 (28)	0.011
G3	1 (17)	5 (83)	
G4	1 (20)	4 (80)	
Depth of infiltration			
pT1	2 (50)	2 (50)	0.897
pT2	9 (53)	8 (47)	
pT3	9 (60)	6 (40)	
pT4	0 (0)	0 (0)	
Nodal stage			
pN0	4 (80)	1 (20)	0.577
pN1	6 (50)	6 (50)	
pN2	4 (44)	5 (56)	
pN3	6 (60)	4 (40)	
Vascular invasion			
Positive	4 (67)	2 (33)	0.688
Negative	12 (50)	12 (50)	
Unknown	4 (67)	2 (33)	
Lymphatic invasion			
Positive	8 (57)	6 (43)	0.577
Negative	0 (0)	0 (0)	
Unknown	12 (55)	10 (45)	
Perineural invasion			
Positive	7 (64)	4 (36)	0.855
Negative	12 (52)	11 (48)	
Unknown	1 (50)	1 (50)	
Metastatic disease			
Positive	6 (55)	5 (45)	0.941
Negative	3 (50)	3 (50)	
Unknown	11 (58)	8 (42)	

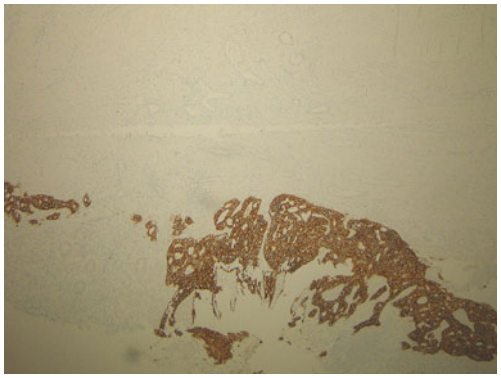

Fig. 2 Example of Her2 heterogeneous gastric carcinoma: <10 % of tumor is strongly positive for Her2; Herceptest 5×

A comparison of the concordance for HER2 over-expression between the whole sections and two different cores, and also for the core with the highest HER2 score, was performed. All results showed an overall concordance between 83.5 % and 86.3 %, with the κ statistics ranging between 0.62 and 0.67, which means substantial agreement. Discrepancies were found mainly in a group of IHC-negative whole sections represented with HER2 2+ cores, which was a consequence of small IHC 2/3+ foci in the overall negatively-scored tumour on the whole section and a low number of positive cells required for scoring bioptic material [16]. In our experience, a somewhat higher percentage of IHC 2+ scores can be expected with TMA technology compared to the rate of 2+ scores on whole sections because of the above-mentioned uneven IHC HER2 reaction. In our study, the percentage of HER2 2+ scored whole sections was 18.7 % (57/305), while the percentage of HER2 2+ cores was 24.4 % (122/499). The percentage of HER2 2+ cores in our study is substantially higher than the percentage reported recently by Park et al. [22], who found only 2.7 % and 4.8 % of HER2 2+ cores in their TMA study, and Kunz et al. [21], who found 7 % of HER2 2+ cores. However, such differences might be explained by the different scoring system used or the different tissue core diameter encountered in the study. While Park et al. [22] used a 10 % cut-off for IHC scoring in cores, TMA

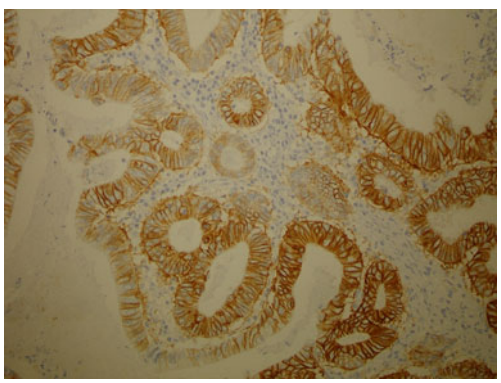

Fig. 3 Uneven immunohistochemical reaction for Her2 protein not only in different areas of tumor but also inside the same tumor gland in tumor that was scored as overall 3+; Herceptest 10×

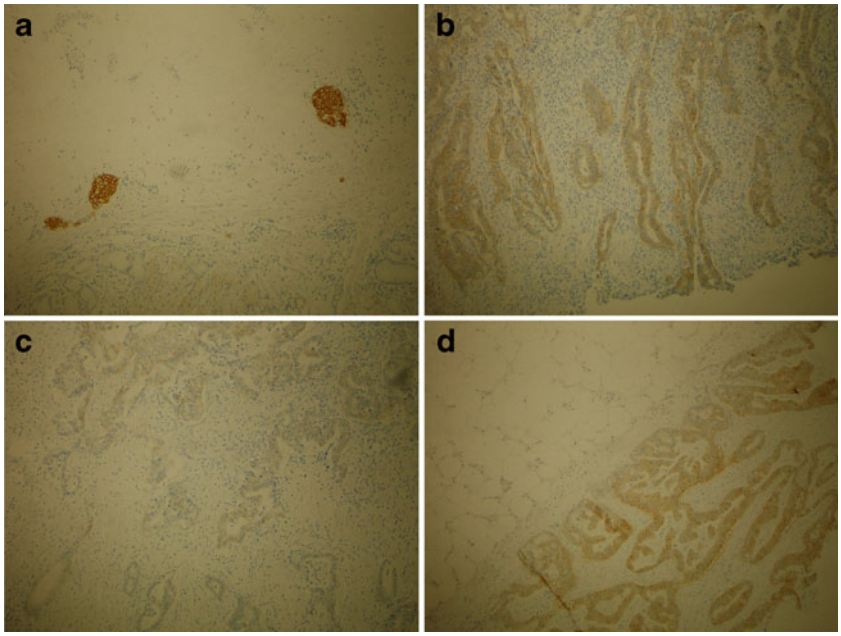

Fig. 4 Fixation artifact? producing negativity of the central part of the tumor in Herceptest; **a** H&E, 10×; **b** luminal (Herceptest, 10×) and (**d**) serosal (Herceptest,10×) parts of the tumor with 2+ positive staining; **c** central part of the same tumor with faint to negative reaction (Herceptest, 10×)

cores were tested analogous to biopsies in our study. In the study by Kunz et al. [21], tissue cores measured only 1 mm compared to 2-mm cores that were used in our study. However, it is still necessary to determine the extent to which the pre-processing issues are responsible for the observed immunohistochemical "heterogeneity" of HER2 in GC. Fixation of GC specimens is difficult to standardise, as formalin penetration varies drastically between small biopsies and large resection specimens. At least a part of the observed IHC HER2-uneven reactions [23, 24] could very likely be explained by fixation artifacts, especially in the case of TMAs where the tissue cores may have been taken from a deeper, less well-preserved tissue. For that reason, in our opinion, the HER2 assessment of TMAs cannot be entirely equal to small endoscopic biopsies [21].

As in the previous studies, there is an excellent concordance between the IHC 3+ and HER2 amplification (100 %) and the IHC 0/1 and lack of HER2 amplification (95.8 %) in the tissue cores used in our study. All IHC 3+ cores (n=41) showed HER2 amplification, mostly of a high-grade type (38 from 41; 92.7 %). In a group of IHC2+ cores, only 14 of them (11.5 %) showed amplification, mainly of a low-grade type (11/14, 78.6 %), while in Park's study [22], 62–96 % of IHC HER2 2+ cases showed amplification. Such a huge difference is a consequence of discrepant HER2 2+ parts in our studies. Only 4.2 % (14/336) of the IHC-negative cores showed amplification, all of them being of a low-grade type. Similar observations about the correlation between the level of amplification and the IHC score were reported recently by Rüschoff's and Park's groups [16, 22].

Comparison of HER2 amplification in the cores' pairs showed a rather homogenous distribution of both HER2 amplification (19 pairs with HGA and 13 with LGA) and non-amplification (194 pairs). Only 3 core pairs showed a discrepancy, with one core being entirely non-amplified, and another one showed foci of HGA. Re-examination of the corresponding whole-section IHC revealed tumors, in which IHC HER2 3+ foci represented 10–20 % of tumours. These findings are similar to the findings of Moelans et al. [24] and Kunz et al. [21], who found no CISH and FISH heterogeneity in spite of present IHC "heterogeneity", using TMA technology. It has to be emphasised, however, that with the TMA approach, the possibility of omitting small amplified tumour cell foci cannot be completely ruled out, either because of coincidental sampling in the cores or non-recognition of small amplified foci in FISH, especially in the case of LGA. Thus, since the current study did not make a comparison of FISH between whole sections and tissue cores, the results should be interpreted with caution. Since several new methods for evaluating the HER2 amplification status (CISH, dual colour CISH, SISH, dual colour SISH) have been introduced in addition to FISH [24], and due to the fact that these methods have some advantages over

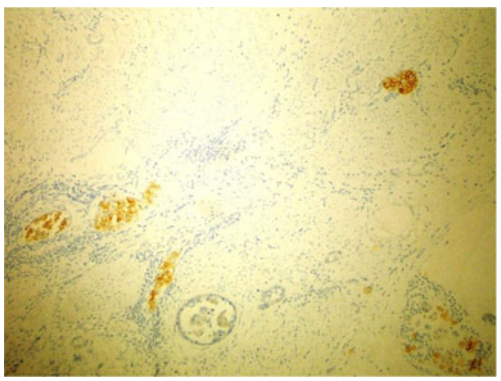

Fig. 5 IHC Her2 3+ positive tumorous thrombi in submucosal lymphatic vessels, in the same paraffin block tumor tissue was completely IHC Her2 negative

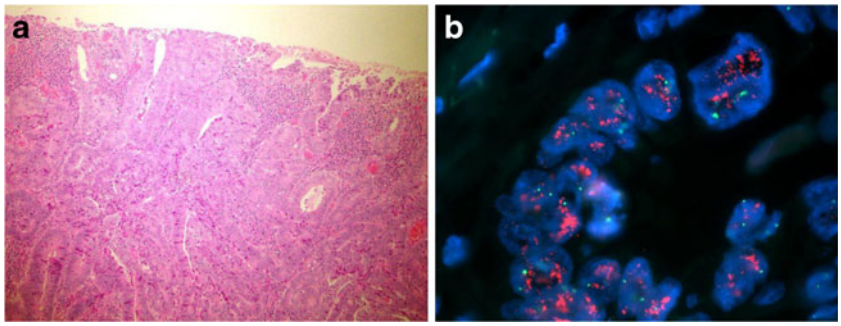

Fig. 6 Moderately differentiated tubular carcinoma with HGA: **a** H&E, 10×; **b** Her 2 FISH

FISH (first of all, the morphology of the tumour can be observed by using a regular bright-field microscope and without the decay of signals), further studies are needed for the comparison of the ISH status of cores and the corresponding whole sections. Still, the TMAs composed of cores from several different tissue blocks of one cancer may be much more appropriate for molecular analysis of potentially heterogeneous targets than the traditional whole-section approach [26, 27]. Considering the fact that there are still no guidelines available regarding the number of tumour blocks to be tested for HER2 in GC, the TMA technology could enable an easier analysis of more than one representative tumor block. In one of our tested tumors, GC in studied paraffin block was completely HER2 negative, while were submucosal lymph vessels filled with HER 2 3+ positive tumor thrombi (Fig. 5).

In this study and according to the European Medicines Agency (EMA) Guidelines [28], the HER2-positive rate of 14.4 % is somewhat lower than recorded in the overall ToGA study (22.1 %). It should be noted, however, that the ToGA study [29] included FISH-positive cases irrespective of the IHC score, as well as IHC 3+ cases. When the EMA criteria were applied retrospectively to the ToGA cohort, the HER2-positive rate of 16 % was comparable to our study and most other published studies [11, 20].

Comparable to some other previous publications, we have found a statistically significant correlation between HER2 over-expression and tumour location [29], the intestinal histologic type [15, 17, 28, 29], tubulopapillary type in the WHO classification [19] and well- to moderately-differentiated tumours [19, 31]. Distribution of the over-expressed tumour types according the Lauren classification (intestinal:diffuse: mixed=34 %:4 %:20 %) was similar to the one reported in the ToGA study [15, 29]. Like some other authors [12, 14, 17], we found an association of HER2 positivity with the clinical outcome with regard to the amplification status. The statistically significant difference was found between the amplified and the non-amplified cases.

To the best of our knowledge, a comparison of the clinicopathological findings between GC with LGA and GC with HGA has not been reported yet. In a group of 36 amplified cases, 20 with HGA and 16 with LGA, a statistical analysis showed that HGA was more frequent in well- to moderately-differentiated papillary and tubulopapillary cancers (Fig. 6), whereas LGA appeared more often in signet ring cell carcinoma and in high-grade tumours, respectively (Fig. 7). Our findings indicate that the level of amplification, and not only amplification per se, may influence the overall survival. Interestingly, patients with HGA, in whom better-differentiated carcinomas and less-aggressive histological types were present, had a somewhat shorter overall survival than patients with LGA, although the difference was not statistically significant. However, these findings should be confirmed with a larger cohort of GCs with HER2 amplification.

Both over-expression and amplification of HER2 were found in 3 mixed-type carcinomas. One carcinoma had strong over-expression (3+) and high-grade amplification of HER2 in the intestinal component, while the diffuse component was negative. The second and the third one had moderate over-expression of HER2 (2+) and low-grade amplification in both the intestinal and diffuse components. Contrary to the results reported by Barros-Silva et al. [30], who found over-expression and amplification in both histological components, these data might indicate that HER2 amplification is not necessarily an early genetic alteration in the carcinogenesis, or that original carcinoma cells originating from a common stem cell might undergo distinct carcinogenic routes, resulting in morphological distinction and different over-expression and amplification of HER2 [32].

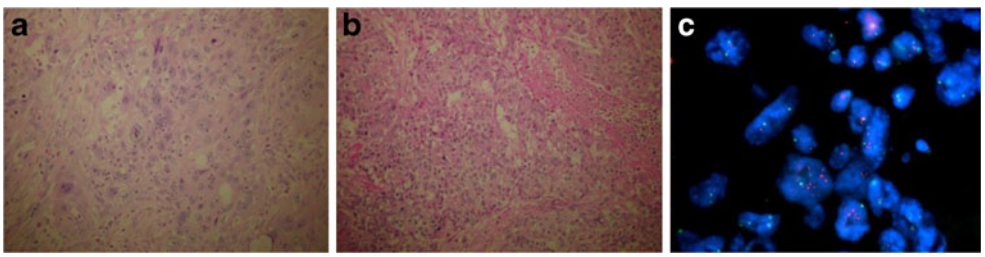

Fig. 7 Anaplastic and poorly differentiated gastric carcinomas with LGA: **a** anaplastic carcinoma H&E, 20×; **b** poorly differentiated carcinoma, H&E, 20×; **c** Her2 FISH

In conclusion, this TMA study showed good concordance of the IHC HER2 results between tissue cores in the TMA and whole sections and an excellent concordance of the IHC and the FISH results for tissue cores. At least a part of the observed IHC HER2 heterogeneity could very likely be explained by fixation artifacts. In addition, adequate fixation can result in a higher concordance for IHC HER2 between the cores and the whole sections. Since HER2 IHC analysis of small samples of a large tumour could eventually lead to underestimation of the level of HER2 protein expression and amplification, and since there are still no guidelines available regarding the number of tumour blocks to be tested for HER2 in GC, the TMA approach could enable an easier analysis of more than one representative tumour block, but more studies are necessary to confirm this.

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Conflict of Interest All authors confirm that they have no conflict of interest to declare.

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