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SUMMARY

Introduction

HER2 gene amplification has been detected in 10% - 20% in gastric adenocarcinomas. In view of the recently demonstrated clinical benefit of the anti-HER2 drug trastuzumab in the treatment of advanced gastric cancer, reliable HER2 testing is of key importance.

Aim

To examine HER2 status in gastro-esophageal adenocarcinomas comparing SP3 and 4B5 immunohistochemistry (IHC) with dual probe HER2 (FISH and SISH).

Methods and results

IHC and SISH was carried out on biopsies of 146 patients with adenocarcinomas of the esophagus and stomach. All SP3-IHC-positive cases, and 91% of 4B5-IHC-positive cases were amplified. Sensitivity of SP3-IHC-positivity and 4B5-IHC-positivity for amplification was 77% and 96%, respectively. Results of FISH performed in 42 cases were identical to SISH. Amplification was heterogeneous in 73% of the adenocarcinomas. 24% of the esophagogastric carcinomas and 7% of distal stomach tumours were amplified.

Conclusions

HER2-positivity is present in a significant proportion of esophago-gastric adenocarcinomas (24%) but at a lower rate in the distal stomach (7%). Sensitivity for amplification is higher with 4B5 IHC than with SP3. FISH and SISH yield identical results, but assessment is much easier with SISH. Our findings provide important guidance for HER2-testing in gastroesophageal adenocarcinomas for patients where anti-HER2 treatment is considered.
Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| ASCO         | American Society of Clinical Oncology |
| CAP          | College of American Pathologists |
| Chr17        | Chromosome 17 |
| CISH         | Chromogen in situ hybridisation |
| EGJ          | Esophagogastric junction |
| EGR          | Esophagogastric region |
| FDA          | Federal Drug Agency |
| FISH         | Fluorescence in situ hybridisation |
| HE           | Haematoxylin Eosin |
| HER1         | Human epidermal growth factor receptor 1 |
| HER2         | Human epidermal growth factor receptor 2 |
| H-score      | Histoscore |
| IHC          | Immunohistochemistry |
| ISH          | In situ hybridisation |
| NA           | Numerical Aperture |
| NPV          | Negative predictive value |
| PPV          | Positive predictive value |
| SISH         | Silver in situ hybridisation |
| TMA          | Tissue micro array |

Key words

- Anti-HER2 therapy
- Esophageal adenocarcinoma
- Gastric adenocarcinoma
- HER2
- Immunohistochemistry
- In situ hybridisation
INTRODUCTION

Therapies directed against tumors overexpressing the transmembranous HER2 receptor as a result of HER2-amplification has become widely available in the last decade for breast carcinomas. HER2-positivity is reported in other carcinomas, most notably gastric and esophageal adenocarcinomas. A large phase III trial employing trastuzumab, directed against the HER2 protein, has been conducted for advanced gastric carcinomas showing clinical benefit (ToGA trial).

Adenocarcinomas of the distal esophagus, esophago-gastric junction (EGJ) and gastric cardia carcinomas share many risk factors and the incidence of these tumors has risen dramatically in the developed world. On the other hand, gastric carcinomas situated in the body or antrum are epidemiologically and biologically distinct from adenocarcinomas situated at or near the EGJ. While the incidence of distal gastric carcinomas is decreasing in industrialized countries, they still constitute a major global health problem. A significant proportion of patients with distal esophageal or gastric carcinomas presents in an advanced disease stage resulting in poor overall survival. A first trial with anti-HER2 therapy in advanced gastric adenocarcinoma showed clinical benefit and with other ongoing trials in advanced gastric and esophageal adenocarcinomas, reliable HER2 status assessment in both esophageal and gastric adenocarcinomas is likely to become as important as in breast cancer.

HER2 status is usually determined by immunohistochemistry (IHC) and/or in situ hybridisation (ISH). With IHC the original 4-tiered scoring system originally described for the FDA-approved HercepTest™ (Dako, Glostrup, Denmark) is widely used irrespective of the IHC-method employed. Samples scored as 0 and 1+ are negative, 2+ as equivocal and 3+ as positive. In the original algorithm for breast cancer, only cases with 2+ score had to be retested with ISH. However CAP/ASCO guidelines require in house validation of 1+ and 3+ samples with ISH before a certified laboratory can confine ISH retesting to 2+ samples.

Recently, a modification of the HercepTest™ scoring system for gastric carcinomas was proposed. The original system required circular staining for a 2+ / 3+ score, and staining of > 10% tumor cells in breast cancer. As noncircular basolateral IHC staining was frequently observed in gastric carcinomas, as well as strong (3+) staining of < 10% tumor cells in biopsies, these elements were added to the original HercepTest™ system.
Novel rabbit monoclonal HER2 antibodies have been recently introduced claiming higher avidity and lower background staining. The 4B5 antibody (Ventana Medical Systems, Tucson, AZ) is directed against the extracellular domain of the HER2-receptor and is FDA-approved. Another antibody is SP3 (Labvision, Thermo Fisher Scientific, Fremont, CA) directed against the intracellular domain providing clearer staining but possibly lower sensitivity. Both antibodies claim an excellent correlation with ISH.

The PathVysion® fluorescence FDA-approved ISH (FISH, Abbott, Abbott Park, IL) is the classic in situ hybridisation test using probes for HER2 and chromosome 17 concomitantly on one slide, allowing for the calculation of a HER2:Chr17 ratio. Dako PharmDx™ FISH used for HER2 testing in the ToGA trial uses a similar approach. FISH requires a fluorescence microscope and assessment in biopsies with heterogenous staining patterns can be very laborious. ISH methods allowing traditional transmitted light microscopy were recently introduced. The dual probe Silver in situ hybridisation (SISH INFORM®, Ventana) uses 2 separate slides for the HER2 and Chromosome 17 probes which allows for a computed HER2:Chr17 ratio. Excellent FISH / SISH correlation is claimed.

No results have been previously published using SP3 and/or 4B5 IHC or SISH in gastric or esophageal adenocarcinomas. We conducted a single institution study in 146 patients using the 2 antibodies with SISH. In addition, all cases showing 1+ immunoscore or higher were retested with Dako FISH. The objective was to determine the predictive value of both antibodies for and the incidence of HER2-amplification.
PATIENTS AND METHODS

The study includes biopsies of 178 consecutive patients with the diagnosis of adenocarcinoma of the stomach or distal esophagus (study period 1999 – 2007). Sufficient material for immunohistochemistry (IHC) and in situ hybridisation (ISH) studies was available in 146 cases with formalin-fixed, paraffin-embedded primary tumour biopsies. The average number of biopsies per case was 5.8 (range, 2 – 14, S.D. 2.4). Location of the tumor was noted (distal esophagus, gastric cardia, body, or antrum).

Esophago-gastric Region (EGR) was defined as the distal esophagus, the esophago-gastric junction or gastric cardia. Distal stomach was defined as gastric body or antrum. On the newly cut slides stained with haematoxylin-eosin (HE), the tumor was typed as ‘intestinal’, ‘diffuse’, ‘mucinous’ or ‘mixed’ using the Laurén classification.

HER2 IHC studies were carried out on the NeXes stainer (Ventana) using 3 µm slides after antigen retrieval (10 mM citrate, pH=7.3, boiling time 25 min.). On all slides, 3 breast cancer tumor samples with an immunoscore of 0, 1+ and 3+ were used as controls. The rabbit monoclonal antibody SP3 was used in a dilution of 1:20 with a 32 min incubation time at 37ºC. Subsequently, iView DAB (Ventana) was carried out for visualisation. In our practise, 300 cases of breast cancer specimens were compared with this SP3 protocol in comparison with DAKO FISH (protocol: see below) yielding excellent concordance. Similarly, 4B5 was applied in prediluted form as provided by the manufacturer.

For scoring of HER2 immunoreactivity the modified Herceptest™ 4-tiered scoring system developed for gastric adenocarcinoma by Hofmann was used. Score: ‘0 (negative)’: no staining or membrane staining in < 10% of cells; ‘1+ (negative)’: faint/barely perceptible membrane reactivity in >10% of cells; cells are only stained in part of their membrane; ‘2+ (equivocal)’: weak to moderate complete or basolateral reactivity in > 10% of tumour cells; ‘3+ (positive)’: moderate to strong complete or basolateral reactivity of > 10% of tumour cells. Cohesive IHC 3+ clones irrespective of the proportion of staining cells, i.e. < 10% are also considered positive as only biopsy material was examined. The number of biopsies, the number of positive biopsies, and HER2 Immunoreactivity expressed as a percentage of total tumor present was noted. A Histoscore (H-score) was calculated: HER2 H-score= [percentage immunoreactive tumor cells (0-100%)] * [Immunoscore (0,1,2 or 3)]. Background staining was noted in 4B5 immunostaining; ‘strong’ background was specified as both nuclear and diffuse cytoplasmic staining, and ‘light’ background as diffuse cytoplasmic immunoreactivity only.
Silver in situ hybridisation (SISH) was studied in all cases using the INFORM HER2 DNA (Ventana) kit with HER2 and chromosome 17 probes on separate serial sectioned 4 µm slides with 8' and 12' pepsin pretreatment, respectively. SISH was carried out using the Benchmark XT stainer (Ventana) with an otherwise standardised protocol supplied by the manufacturer.

FISH was carried out with the HER2 FISH PharmDx™ kit (Dako) in 40 cases with SP3 and/or 4B5 HER2-immunoreactivity of 1+ and higher; 2 cases with only strong background staining in 4B5 immunohistochemistry were added. 3 µm slides were deparaffinized, placed during 10 min in pretreatment solution at 95°C, and after 15 min cooling at room temperature (RT) put in wash buffer 2 times for 3 min. Then, pepsine was applied for 6 min at RT and the washing step was repeated. After dehydration in graded ethanol, slides left to dry at RT for 6 min, and after addition of 10 µl HER2/Chr17 probe mix a coverslip with sealant was applied and slides were placed in the ThermoBrite Hybridizer (StatSpin, Norwood, MA, USA). Denaturation step was 5 min at 82°C followed by overnight hybridisation at 45°C. Then, slides were retrieved from the Hybridizer, coverslips were removed, and stringent wash at 65°C was carried out followed by wash buffer treatment. Finally, mounting medium was applied with a new coverslip. Slides were stored at -20°C until assessment.

Assessment of in situ hybridisation was performed on a Zeiss Axioscope 40 fluorescence microscope with 40x/N.A.1.3 and 100x/N.A.1.3 (oil) objectives. SISH slides were counted using same objectives but with bright field light. Before ISH assessment the serial slides stained with HE, SP3 and 4B5 immunostains were re-evaluated. In the ISH slides, all tumour areas were assessed. Areas containing the highest HER2 counts were preferentially assessed by counting HER2 and Chromosome 17 (Chr17) signals in at least 20 nuclei. HER2:Chr17 ratios were calculated. When counts exceeded 300 per 20 nuclei, ‘>300’ was noted, and a HER2:Chr17 ratio of > 6 was assumed. Positive ISH was defined as a HER2:Chr17 ratio of ≥ 2.2. Polysomy was defined as an average of ≥ 3 chromosomes 17 per nucleus. Of all slides with dual (SISH and FISH) assessments, photomicrographs were taken and stored in Research Assistant 5 (RVC, Baarn, The Netherlands).
RESULTS

Tumor characteristics (table 1)

Histologic characteristics and the anatomical location of the 146 gastro-esophageal tumours are presented in table 1. Biopsies of 72 adenocarcinomas were from the Esophago-Gastric Region (EGR); 44 (61%) from the distal esophagus and 28 (39%) from the gastric cardia. 63 were classified as intestinal type, 6 as mixed and 3 as mucinous. 74 adenocarcinomas were from the distal stomach; 24 (32%) in the gastric body and 50 (68%) in the antrum. 54 were of intestinal type, 9 mixed, 9 diffuse and 2 of mucinous type.

HER2 immunostains (table 2)

SP3 immunoreactivity (figure 1) was completely absent in 125 (86%) cases. An immunoscore of 1+ was seen in 4, 2+ in 6 and 3+ in 11 cases. Membranous 4B5 immunoreactivity (figure 2) was absent in 106 (72%) cases; immunoscores of 1+ were present in 17 cases, 2+ in 6 and 3+ in 17. All cases with immunoscores of 1+ and higher showed immunoreactivity in at least 10% of tumor cells. U-shaped basolateral staining was quite often noted. Background staining was entirely absent with the SP3-antibody. The 4B5 stains showed invariably extensive cytoplasmatic background staining of the gastric foveolar layer (figure 5A); intestinal metaplasia when present also showed this staining pattern but less substantial. In addition, cytoplasmatic background staining of tumor was noted in 40 (27%) cases, of which 7 (5%) strong with nuclear immunoreactivity. In additional 2 cases, only strong background staining was noted without notable membranous staining (figure 5B).

HER2 SISH / FISH versus Immunoscore (tables 3A and 3B)

SISH was performed in all 146 cases (table 3A; figure 3). In some cases of SISH some nuclear haze was present, possibly as a result of fixation time of approximately 6h (figure 5C). All 17 cases with SP3 immunoscores 2+/3+ were amplified as were 5 out of 129 (4%) cases with negative SP3 immunohistochemistry (immunoscores 0/1+). Only 1 case out of 123 with 4B5 immunoscores 0/1+ was amplified, and 21 out of 23 (91%) cases with immunoscores 2+/3+. The 2 amplified cases with SP3-immunoscore 0 were scored 2+ in the 4B5 immunohistochemistry. The 3 amplified cases with...
SP3-immunoscore 1+ were 2+ in the 4B5 in 2 cases, and 1+ in one case. Two cases with strong 4B5 background staining of nuclei (but no membranous staining) were not amplified. Sensitivity for amplification was 77.3% for SP3 and 95.5% 4B5 ‘IHC positive’ cases as defined by immunoscores 2+/3+. Specificity was 100% for SP3-positive IHC and 98.4% for 4B5-positive IHC, respectively. The positive predictive value (PPV) of SP3 positivity was 100% and the negative predictive value (NPV) 96.1%. For 4B5 the PPV was 91.3% and the NPV 99.2%.

Of 42 performed FISH procedures (figure 4) the results were identical to SISH. 22 cases were amplified (table 3B). Granular red background staining was seen in a minority of cases in both tumor and normal epithelial cells (figure 5D).

**Heterogeneity of HER2 positivity (table 4)**

Heterogeneity of HER2-immunoreactivity was the dominant pattern, and areas of HER2 amplification closely matched positive HER2-immunoreactivity. Percentage of tumor staining for an antibody without regard of immunoscore, the number of positive biopsies and/or calculated H-score did not predict amplification status. The only generalisation that could be made was that most amplified cases had a 2+/3+ immunoscore in SP3, and 3+ (any percentage) or 2+ (>50% tumor area) in 4B5 IHC.

**Polysomy**

Polysomy defined as ≥3 chromosome 17 copies per cell was present in 28 cases (19.2%). Polysomy was noted in 7 out of 22 (32%) amplified cases showing invariably ≥5 HER2 signals per nucleus. In contrast, polysomy present in 21 out of 104 cases had <5 HER2 signals per nucleus. Therefore, assessment of polysomy – quite often a striking finding in tumours in our study – did not contribute to the prediction of amplification.

**HER2 amplification and anatomical location/histological type (table 5)**

Of the total of 117 adenocarcinomas of the intestinal type, 20 (17%) showed HER2-amplification; 17 cases were located in the EGR and 2 in the distal stomach. Two out of 15 cases (13%) with mixed histology were also amplified located in the distal stomach. None of the 14 cases with diffuse or mucinous types showed amplification.
Of the tumors situated in the EGR, 17 (24%) were HER2 amplified: 12 out of 44 distal esophageal adenocarcinomas and 5 of 28 cardia carcinomas. In the distal stomach, 5 (7%) showed HER2 amplification: 2 of 24 body and 3 of antrum carcinomas.
DISCUSSION

In a study of 146 cases with biopsies of primary esophagus and gastric adenocarcinomas, frequent HER2-immunoreactivity using 2 novel rabbit monoclonal antibodies was identified. Both SP3 and 4B5 immunostaining had a high positive predictive value for HER2-amplification. 4B5 had a much higher negative predictive value than SP3. SISH – when compared with FISH in selected cases - yielded identical results compared with FISH. HER2-amplification was most often present in biopsies of esophagus and gastric cardia adenocarcinomas (27% and 18%, respectively); amplification was less frequent in adenocarcinomas of the body and antrum region (7%).

Many studies have been published on the concordance of HER2 immunohistochemistry and in situ hybridisation in breast cancer, usually focusing on the rabbit polyclonal antibody A408 (used in HercepTest®) or the mouse monoclonal CB11 (used in the previous generation of Ventana Pathway®), both FDA-approved kits. In daily practice the application of these antibodies results in a relatively high percentage of equivocal 2+-immunoscores. In order to achieve an unequivocal 3+-immunoscore pattern in cases with high amplification a high background in normal tissue has to be accepted precluding easy and reproducible assessment of immunohistochemistry. The new generation of more avid rabbit monoclonal antibodies has been developed in order to produce better signal-to-background patterns. In the field of Estrogen Receptor-immunohistochemistry, the new SP1-antibody has become an acclaimed test. SP3 and 4B5 are new rabbit monoclonal antibodies directed against the intracellular and extracellular of the HER2-receptor, respectively.

Ricardo et al reported a significant level of agreement of SP3 and CB11 IHC in breast carcinomas when compared with CISH, and claimed easier assessment of SP3 immunostains. However, of 50 CISH-amplified samples 21 cases showed negative SP3/CB11 IHC scores. Nunes et al reported a better negative predictive value of SP3 immunohistochemistry, with CB11 being slightly inferior but Herceptest equivalent to SP3. In a recent update of UK recommendations on HER2 testing, Walker et al. reported equivalent results of 4B5 in comparison with Herceptest which is in concordance with a report of the quality assurance program Nordiq.

In our study, both the SP3 and 4B5 yielded good correlations with both in situ hybridisation methods. SP3 IHC produced clear immunostains facilitating rapid and easy assessment. However, 5 out of the 22 amplified cases were SP3-IHC negative. 2 (out of 21) cases with a complete negative staining...
pattern and 3 (out of 4) cases with 1+ pattern. The 4B5 antibody exhibited different staining characteristics: only 1 (out of 19) negative in IHC (1+ score) case was amplified, as were 20 (out of 22) cases with positive IHC (score 2+/3+). The 4B5 was considerably more sensitive than the SP3 with a better correlation with *in situ* hybridisation. However, some drawbacks in the 4B5 IHC were apparent: distinct cytoplasmic background staining was present almost invariably in the foveolar layer of the stomach; in addition, strong cytoplasmatic and even nuclear background could be seen in some tumor areas. No amplification was present in the foveolar or tumor cells exhibiting the background staining but assessment of IHC was occasionally hindered by these phenomena which are not seen in breast pathology.

Due to recent introduction few studies comparing 4B5 with other antibodies have been published in breast carcinomas. Powell et al.\(^3\) from Ventana reported sharper membrane staining, less background and better concordance with FISH using 4B5 in 322 breast carcinomas in comparison with CB11. Egervari et al.\(^3\) comparing 6 HER2 antibodies in a tissue micro-array composed of 199 breast cancers with FISH, *In this array, amplification was present in 23 cases.* In contrast to our findings, they found *that some cases with immunoscores of 0/1+ with 4B5 IHC showed amplification.* This underscores the need for in house validation of immunohistochemistry prior to limiting *in situ* hybridisation to the category of 2+ immunoscores as required by 2007 ASCO/CAP HER2 guidelines for breast cancer\(^25\).

Reported frequencies of positive HER2-immunoreactivity in gastro-esophageal cancer vary extensively. Table 6 lists the studies with full-paper reports in which *in situ* hybridisation was carried out in more than 50 cases. A comprehensive review by Hofmann et al.\(^3\) described 3,264 cases of gastric adenocarcinoma showing a positivity rate of 17.6% defined by IHC. In our selection of ISH-confirmed HER2 status in table 6, this percentage is lowered to 12%. Even after detailed review of the full-paper reports it remains unclear if adenocarcinomas of the gastric cardia are included in the studies listed in table 6, let alone the relative contribution of these tumors to overall findings. Thus, our finding of a 7% HER2-positivity rate in adenocarcinomas of the distal stomach is no outlier; if cardia carcinomas are included, a 11% positivity rate is reached in the present study. Bang et al. claims a 22.1% HER2-positivity in 3,280 cases of advanced, recurrent and/or metastatic gastric adenocarcinomas *as defined by either 3+ immunoscore using HercepTest\(^\text{TM}\) or amplification with Dako FISH in an abstracted ToGA-trial report*. Adenocarcinoma of the stomach and the gastro-esophageal
junction had a HER2-positivity of 20.9% and 33.2%, respectively. This is substantially higher than most reports including the present study. In contrast to the present study, they found 4.9% FISH positive cases in which IHC was completely negative, and a 15% amplification rate when immunoscore was 1+. Subsequent analysis failed to show clinical benefit of trastuzumab in the FISH-positive but immunonegative subgroups. Another possible explanation might be that patients with advanced disease only were accrued for the ToGA trial while HER2-amplification could be to be a late event in oncogenesis. The average HER2-positivity rates of the patients included in the ToGA at the time of the report of Bang et al. did not vary according the geographical region (Europe or Asia) but was a function of histological type in concordance with our study.

The few larger studies with in situ hybridisation confirmed HER2 status in esophageal adenocarcinoma report a HER2-positivity rate of at least 15%. Our 27% HER2 amplification rate is in line with substantial higher HER2-positivity in esophageal carcinoma rather than (distal) gastric adenocarcinoma. Unfortunately, questions concerning definitions, segmentation of oncological entities, division of treatment groups and different research interests hinder a comprehensive view of HER2-positivity rates of the anatomical region comprising the distal esophagus and the entire stomach.

An important difference in HER2 immunoreactivity and amplification between breast cancer and gastro-esophageal adenocarcinomas is the striking heterogeneity of HER2-positivity in the latter. The 2007 ASCO/CAP HER2 Guidelines in breast cancer demands HER2-immunoreactivity/amplification in at least 30% of breast cancer tumor areas in order to render a HER2-positivity as a conclusion. Hofmann et al propose to allow even less than the original HercepTest™ 10% 3+ pattern of immunoreactivity in biopsies of gastric carcinomas; in resection specimens the 10% threshold is retained. The considerable heterogeneity of HER2-positivity – 73% of all HER2-amplified cases in our study – suggests that HER2 amplification is a late event in gastro-esophageal adenocarcinomas.

Nevertheless, a recent report of a large phase III trial using trastuzumab (ToGA trial) showed clinical benefit, especially in the group with both FISH positivity and an immunoscore of at least 2+ using Herceptest. The magnitude of clinical benefit is similar to metastatic breast carcinomas. This is remarkable considering the heterogeneous HER2-positivity in gastric adenocarcinomas in contrast to breast cancer, in which more than 95% of HER2-positive cases are homogeneously HER2-amplified. Recently, Marx et al reported highly homogeneous HER2 amplification in multiple sections obtained...
from 8 highly amplified cancers. Possibly, this is a selection, as they studied 166 surgically resected gastric cancers cases, 27 of which showed unequivocal amplification.

Silver in situ hybridisation is a recently introduced technique being somewhat similar to CISH in that a conventional microscope can be used to assess the probe signals. Reports are still limited\textsuperscript{29, 36} with less than 150 cases examined; so far, good correlations between FISH and SISH are described. In our study, 42 cases yielded identical results using both techniques. Assessment of HER2/Chromosome 17 signal in the multiple biopsies was much faster and easier with SISH in comparison with FISH, and allows good correlation between IHC and SISH using conventional techniques as ink-marking.

Most contemporary studies outside trials use the very cost-effective tissue micro-array (TMA) technique. However, given the high incidence of heterogenous HER2-immunoreactivity/amplification, we felt that using the TMA technique in this field would yield an underestimation of the incidence of HER2-amplification rates. While we did not study resection specimens, the claim of Marx et al.\textsuperscript{1} that HER2 amplification is highly homogenous is contradicted by our findings in biopsy material.

In conclusion, HER2 amplification is present in a significant proportion of esophago-gastric region adenocarcinomas (24%) but at a much lower rate in the distal stomach (7%). Both rabbit monoclonal antibodies SP3 and 4B5 can be used for initial screening for possible amplification though the 4B5 antibody has the highest negative predictive value. FISH and SISH yields identical results. The SISH assay offers relatively easy and fast assessment. Our findings provide important guidance for HER2-testing in gastroesophageal adenocarcinomas for patients where anti-HER2 treatment is considered.
DISCLOSURE / CONFLICT OF INTEREST

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### Tables and Figures

Table 1. Histologic classification and anatomical position of the adenocarcinomas.

| Laurén classification | Total esophagus | Total cardia | Total EGR | Total body | Total antrum | Total distal stomach |
|-----------------------|-----------------|--------------|-----------|------------|--------------|----------------------|
| intestinal            | 40 (91%)        | 23 (82%)     | 63 (88%)  | 15 (62%)   | 39 (78%)     | 54 (73%)             |
| mixed                 | 3 (7%)          | 3 (11%)      | 6 (8%)    | 6 (25%)    | 3 (6%)       | 9 (12%)              |
| diffuse               | 1 (2%)          | 2 (7%)       | 3 (4%)    | 3 (13%)    | 6 (12%)      | 9 (12%)              |
| mucinous              | 0 (0%)          | 0 (0%)       | 0 (0%)    | 0 (0%)     | 2 (4%)       | 2 (3%)               |
| total                 | 44 (100%)       | 28 (100%)    | 72 (100%) | 24 (100%)  | 50 (100%)    | 74 (100%)            |

EGR: esophagogastric region
Table 2. SP3 versus 4B5 HER2 immunoscores.

| SP3 | 0   | 1+  | 2+  | 3+  | Total |
|-----|-----|-----|-----|-----|-------|
| 0   | 105 | 15  | 5   | 0   | 125 (86%) |
| 1+  | 1   | 2   | 1   | 0   | 4 (3%)   |
| 2+  | 0   | 0   | 0   | 6   | 6 (4%)   |
| 3+  | 0   | 0   | 0   | 11  | 11 (7%)  |
| total| 106 | 17  | 6   | 17  | 146 (100%) |

Deleted: 0
Deleted: 2
Deleted: 1
Deleted: 5
Deleted: 7
Table 3A. HER2 SP3 and 4B5 immunoscores versus Silver in situ hybridisation (SISH)

| SP3 | neg  | pos  | total |
|-----|------|------|-------|
| 0   | 123  | 2    | 125   |
| 1+  | 1    | 3    | 4     |
| 2+  | 0    | 6    | 6     |
| 3+  | 0    | 11   | 11    |
| total| 124 | 22   | 146   |

Table 3B. HER2 SP3 and 4B5 immunoscores versus FISH

| SP3 | neg  | pos  | total |
|-----|------|------|-------|
| 0   | 19   | 2    | 21    |
| 1+  | 1    | 3    | 4     |
| 2+  | 0    | 6    | 6     |
| 3+  | 0    | 11   | 11    |
| total| 20  | 22   | 42    |

| 4B5 | neg  | pos  | total |
|-----|------|------|-------|
| 0   | 106  | 0    | 106   |
| 1+  | 16   | 1    | 17    |
| 2+  | 2    | 4    | 6     |
| 3+  | 0    | 17   | 17    |
| total| 124 | 22   | 146   |
Table 4. SP3 and 4B5 immunoscores versus percentage IHC positive cells in amplified/nonamplified tumors

|       | % pos cells in IHC |       | % pos cells in IHC |
|-------|--------------------|-------|--------------------|
|       | 1-                 | 11-   | 51-               | 76-               | Sub-
|       | 10                | 50    | 75                | 100               | total |
| SP3 0 | 123               | 0     | 0                 | 0                 | 0     | 123   |
| 1+    | 0                  | 0     | 0                 | 0                 | 0     | 0     |
| 2+    | 0                  | 0     | 0                 | 0                 | 0     | 0     |
| 3+    | 0                  | 0     | 0                 | 0                 | 0     | 0     |
| 4B5 0 | 106               | 0     | 0                 | 0                 | 0     | 106   |
| 1+    | 0                  | 3     | 10                | 0                 | 3     | 16    |
| 2+    | 0                  | 0     | 2                 | 0                 | 0     | 2     |
| 3+    | 0                  | 0     | 0                 | 0                 | 0     | 0     |

|       | % pos cells in IHC |       | % pos cells in IHC |
|-------|--------------------|-------|--------------------|
|       | 1-                 | 11-   | 51-               | 76-               | Sub-
|       | 10                | 50    | 75                | 100               | total |
| SP3 0 | 0                  | 1     | 1                 | 0                 | 0     | 2     |
| 1+    | 0                  | 0     | 2                 | 0                 | 1     | 3     |
| 2+    | 0                  | 0     | 2                 | 0                 | 4     | 6     |
| 3+    | 0                  | 0     | 4                 | 0                 | 7     | 11    |
| 4B5 0 | 0                  | 0     | 0                 | 0                 | 0     | 0     |
| 1+    | 0                  | 0     | 1                 | 0                 | 0     | 1     |
| 2+    | 0                  | 1     | 2                 | 0                 | 1     | 4     |
| 3+    | 0                  | 0     | 6                 | 0                 | 11    | 17    |
Table 5. Number (and percentage of biopsies) of HER2 amplified cases versus Laurén classification and anatomical location.

| Laurén Position | Esophagus | Cardia | EGR | Body | Antrum | Distal Stomach |
|-----------------|-----------|--------|-----|------|--------|----------------|
| Intestinal      | 12 (30%)  | 5 (22%)| 17 (27%) | 1 (7%) | 2 (5%) | 3 (6%)         |
| Mixed           | 0 (0%)    | 0 (0%)| 0 (0%) | 1 (17%) | 1 (33%) | 2 (22%)       |
| Diffuse         | 0 (0%)    | 0 (0%)| 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%)        |
| Mucinous        | 0 (0%)    | 0 (0%)| 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%)        |
| Total           | 12 (27%)  | 5 (18%)| 17 (24%) | 2 (8%) | 3 (6%) | 5 (7%)        |
### Table 6. HER2 status in studies with ISH on adenocarcinomas

| Reference   | Method  | n (total) | n (pos) | % (pos) |
|-------------|---------|-----------|---------|---------|
| Stomach     |         |           |         |         |
| Ishikawa    | FISH    | 105       | 19      | 18%     |
| Takehana    | FISH    | 352       | 29      | 8%      |
| Risio       | FISH    | 72        | 11      | 15%     |
| Varis       | CISH    | 52        | 9       | 17%     |
| Tanner      | CISH    | 131       | 16      | 12%     |
| Park        | FISH/CISH | 182    | 7       | 4%      |
| Yano        | FISH    | 200       | 54      | 27%     |
| Kim         | FISH    | 248       | 19      | 8%      |
| Barros-Silva| FISH   | 463       | 38      | 8%      |
| Marx        | FISH    | 166       | 27      | 16%     |
| **Total**   |         | 1,981     | 229     | **mean 12%** |
| Esophagus   |         |           |         |         |
| Brien       | FISH    | 63        | 12      | 19%     |
| Tanner      | CISH    | 100       | 24      | 24%     |
| Reichelt    | FISH    | 110       | 16      | 15%     |
| **Total**   |         | 273       | 52      | **mean 19%** |

FISH: fluorescence in situ hybridisation; CISH: chromogenic in situ hybridisation
FIGURE LEGENDS

**Figures 1 / 2.** Figures 1 (SP3 IHC) and 2 (4B5 IHC) show identical cases. 1A: basolateral staining in SP3 (Score 1+) but in 2A a 3+ staining pattern in 4B5; 1B and 2B: 1+ staining in both SP3 and 4B5; 1C and 2C (low magnification): heterogeneous staining with 3+ pattern in the right half of the tumor and a 1+ staining in the left side, identical in SP3 and 4B5; 1D and 2D: detail of the 1+ / 3+ transition zone.

**Figure 3 / 4.** Figure 3 (SISH) and 4 (FISH) with identical cases. 3A and 4A: high amplification; 3B and 4B: low amplification; 3C and 4C: (lower magnification) area with (white arrow) and without (yellow arrow) magnification; 3D: Chromosome 17 SISH: polysomy with > 3 copies chromosome 17 per nucleus; 4D: polysomy. FISH: red dots = HER2; green dots = Chromosome 17.

**Figure 5.** A: 4B5 IHC: background staining in foveolar layer of gastric epithelium; B: 4B5 IHC: strong nuclear and cytoplasmic background staining in a diffuse type adenocarcinoma; C: HER2 SISH: nuclear haze; D: FISH: red granular background staining of some nuclei (encircled) among nuclei showing high amplification.
FIGURES
3 files in EPS:
Figures 1 and 2 (one page)
Figures 3 and 4 (one page)
Figure 5 (half page)

END OF MANUSCRIPT
Table 4. SP3 and 4B5 in relation to amplification

|                | SP3   | 4B5   |
|----------------|-------|-------|
| concordance    | 96.6% | 97.9% |
| discordance    | 3.4%  | 2.1%  |
| positive predictive value | 100.0% | 91.3% |
| negative predictive value | 96.1% | 99.2% |
| sensitivity    | 77.3% | 95.5% |
| specificity    | 100.0%| 98.4% |

Table 7. Percentage HER2-positivity

| Anatomical position | Total | Total |
|---------------------|-------|-------|
|                     | distal| stomach |
| Laurén              | body  | antrum |
| intestinal          | 30%   | 7%     |
|                     | 22%   | 5%     |
|                     | 27%   | 6%     |
| mixed               | 0%    | 17%    |
|                     | 0%    | 33%    |
|                     | 0%    | 22%    |
| diffuse             | 0%    | 0%     |
|                     | 0%    | 0%     |
|                     | 0%    | 0%     |
| mucinous            | 0%    | 0%     |
|                     | 0%    | 0%     |
|                     | 0%    | 0%     |
| total               | 27%   | 8%     |
|                     | 18%   | 6%     |
|                     | 24%   | 7%     |

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Figures 1 / 2. Figures 1 (SP3 IHC) and 2 (4B5 IHC) show identical cases. 1A: basolateral staining in SP3 (Score 1+) but in 2A a 3+ staining pattern in 4B5; 1B and 2B: 1+ staining in both SP3 and 4B5; 1C and 2C (low magnification): heterogeneous staining with 3+ pattern in the right half of the tumor and a 1+ staining in the left side, identical in SP3 and 4B5; 1D and 2D: detail of the 1+ / 3+ transition zone.

144x219mm (600 x 600 DPI)
Figure 5. A: 4B5 IHC: background staining in foveolar layer of gastric epithelium; B: 4B5 IHC: strong nuclear and cytoplasmic background staining in a diffuse type adenocarcinoma; C: HER2 SISH: nuclear haze; D: FISH: red granular background staining of some nuclei (encircled) among nuclei showing high amplification.

144x236mm (600 x 600 DPI)
NOTE: we discarded original panel 3&4, which is and was in .eps format as required. However, the new panel 3&4 can now not be uploaded for .eps file format was not accepted. We would like to know how we can upload the new panel 3&4. As for now, we have a low resolution net figure 3D as an example to show to the reviewer.

240x199mm (100 x 100 DPI)