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# Hybridization for HER2 testing in gastric carcinoma: a comparison of fluorescence in situ hybridization with a novel fully automated dual-color silver in situ hybridization

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**Running title:** HER2 gastric carcinoma

**Keywords:** HER2; FISH; SISH; gastric carcinoma; dual-color hybridization

## Abstract

*Aims:* Amplification of the human epidermal growth factor receptor 2 (HER2) gene has been reported in gastric carcinoma (GC). Accordingly, trastuzumab plus chemotherapy has recently become the new standard treatment for HER2 positive advanced GCs.

*Methods and results:* We sought to compare the alleged gold standard for hybridization (FISH) with a novel fully automated bright-field dual-color silver enhanced in situ hybridization (SISH) in a series of 166 GC samples. Additionally, tumors with discordant results obtained by FISH and SISH were analyzed by real-time quantitative PCR with the LightMix kit HER2/neu. 17.5% and 21% of the samples were amplified by FISH and SISH, respectively. Heterogeneity was identified in up to 52% of the cases. In 96.4% of cases FISH showed the same results as SISH. All six discordant cases were positive by SISH and negative by FISH. On review of the FISH slides, all contradictory cases were polysomic and were confirmed as negative for amplification by real-time PCR. Interestingly, all ratios in this latter group were between 2.06 and 2.50 so setting the cut-off for amplification at  $\geq 3$  resulted in a perfect concordance.

*Conclusions:* Dual color SISH represents a novel method for the determination of *HER2* status in GC.

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## Introduction

In 1987, Slamon et al. described the relationship between the amplification of the HER2 gene and a group of breast carcinomas<sup>1</sup> for the first time. The development, in later years, of a specific treatment for this alteration, the antibody trastuzumab, has been one of the greatest successes of solid tumor oncology. Naturally, there have been attempts to reproduce this success in other neoplasias which can also show amplification of HER2 (carcinomas of the stomach, bladder, lung etc.).<sup>2,3,4</sup> Finally, in the year 2009, results were presented of the ToGA trial in patients with advanced gastric carcinoma.<sup>2</sup>

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**The ToGA trial was a prospective randomized multicenter phase III trial conducted in 24 centers. This study looked for HER2 expression/amplification in 3807 patients with gastric carcinomas (GC) with a positivity rate of 22% (either by FISH or IHC 3+). Five hundred and eighty four HER2 positive GCs were included and randomized between cisplatin plus fluoropyrimidine (5Fu or capecitabine) (Ch) or the same chemotherapy plus trastuzumab (Ch+T). Patients treated with Ch+T showed a clinically and statistically significant improvement in terms of overall survival (13.8 months vs 11.1 months, HR 0.7 95%CI 0.6 – 0.9, p= 0.0046). Secondary end points of the study were also met; thus Ch+T significantly improved overall response rate and progression-free survival, compared with Ch alone, without any increase in toxic effects including cardiac events. Therefore, trastuzumab plus 5Fu/cisplatin chemotherapy has become the new standard treatment for HER2 positive advanced gastric cancer.<sup>2</sup>**

However, since trastuzumab was first approved for breast cancer, there have been notable inconsistencies in [procedures for studying HER2](#). This “cancer biomarker problem” significantly affected the specific method: immunohistochemistry (IHC), FISH or the various types of brightfield in situ hybridization (ISH). **Similarly it affected** the concordance between techniques; the cut-off point and the work-flow algorithm, although IHC has traditionally been considered the primary testing modality.<sup>5,6,7</sup> The fact that the first ASCO/CAP consensus was not published until 2007<sup>8</sup> and the lack of consensus among different national guidelines <sup>(reviewed in 9)</sup> undoubtedly contributed to the problem. Further fuelling the controversy was the [subtle](#) recommendation of initial FISH in specific settings (core-needle biopsies)<sup>10</sup> and the direct suggestion of “FISH as the primary HER2 testing modality for women with breast cancer who are candidates for HER2 targeted therapies”.<sup>11</sup> This latter paradigm shift has remained largely unnoticed, despite publication in a high impact journal and the fact that Slamon was among its authors more than 20 years after his initial discovery. The situation that we have outlined above has recently been termed “the HER2 testing conundrum” and the result is that as many as one in five HER2 tests give the wrong answer.<sup>12,13</sup>

**However, it must be pointed out that the clinical benefit of trastuzumab in the ToGA trial seems to be restricted to patients IHC 2+ and FISH positive or IHC 3+, when compared with patients IHC 0 or 1+ and FISH negative.**<sup>2</sup>

With the above controversies in mind, we sought to compare the alleged gold standard (FISH) with a novel fully automated brightfield dual-color approach in a series of gastric carcinoma samples.<sup>14</sup> Our aim was to provide robust analytical and post-analytical information in the setting of gastric

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carcinoma to guide the clinical validation of the different assays, as has been suggested.<sup>15</sup>

## Material and methods

### Tumor samples

A randomly selected total of 166 gastric adenocarcinomas from several Spanish institutions were collected for HER2 status analyses. The study was approved by a centralized ethics committee. Before paraffin embedding, tissues were fixed in buffered formalin. We have no data regarding how concentrated the fixative was or the time of fixation. Fifty (30.1%) were endoscopic biopsies and 116 (69.9%) surgical specimens of which 15 (9%) were distant metastases. All cases were diagnosed according to the Lauren's classification<sup>16</sup>: 86 (51.8%) intestinal type, 47 (28.3%) diffuse type and 33 (19.9%) indeterminate type.

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### Analytical Phase

Sections of tumoral tissue samples 4µm-thick were cut and placed on charged polylysine-coated slides for analysis.

### FISH

HER2 copy number was investigated by FISH, using the PathVysion HER2 DNA probe kit (Vysis Inc, Abbot Laboratories, IL), with the DAKO Histology FISH Accessory kit. The manufacturer's instructions were modified using DAKO Histology kit in order to optimize the technique (decreased laboratory processes).<sup>17</sup> Sections were incubated at 56°C overnight, desparaffinized in two series of xylol followed by rehydration with an ethanol series. Slides were

pretreated with Pre-treatment Solution in a water bath at 97°C for ten minutes.

Enzymatic digestion was carried out with Ready-to-Use Pepsin for three minutes at room temperature (endoscopic biopsies) or six minutes at 37°C (surgical specimens). After dehydration with graded ethanol series, 10µl of HER2/CEP17 probe mix was applied to each tissue section. The slides and probe were denaturalized at 80°C for five minutes and hybridized at 37°C overnight in a Dako Hybridizer. On the second day, the sections were washed with Stringent Wash Buffer at 65°C for ten minutes in a water bath. Next, the slides were dehydrated using graded ethanol series and 10µl of fluorescence mounting media containing 4'-6-diamino-2-phenylindole (DAPI) was applied.

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### Dual-color SISH

Automated SISH was performed on Ventana Benchmark XT (Ventana Medical Systems, Tucson, AZ). INFORM HER2 DNA Probe and INFORM Chromosome 17 Probe was visualized on the same slide following manufacturer's protocols with a few variations. Assay conditions were modified for optimal results. The entire assay procedure (deparaffinization, pretreatment, hybridization, stringency wash, signal detection and counterstaining) was fully automated.<sup>14</sup> Pretreatment was performed with Reaction Buffer and enzyme digestion with ISH Protease 3 for 12 minutes. HER2 probe was denatured at 95°C for 15 minutes and hybridized at 56°C for six hours. Chromosome 17 centromere probe was denatured at 95°C for 12 minutes and hybridized at 44°C for three hours. Stringency washes for HER2 probe were performed at 72°C for eight minutes (three steps) and incubated with anti-DNP (dinitrophenol) antibody for 20 minutes. Next, tissue slides were incubated with HRP-conjugated anti-rabbit

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antibody for 16 minutes. The silver signal for HER2 was revealed by sequential silver reactions (Silver C's incubation time, 4 minutes). For chromosomal 17 centromere probe, three stringency washes were applied at 59°C for eight minutes. Next, tissue slides were incubated with anti-DNP antibody for 20 minutes and with alkaline phosphatase-conjugated antibody for 12 minutes. The signal of centromere was visualized with Red ISH Naphthol reaction for four minutes. The tissues were counterstained with Hematoxilin II for eight minutes and Bluing Reagent for four minutes. The slides were covered with Cytoseal mounting media. Some of the slides had to be stained once or twice. We were not able to identify the cause of these failures, which other authors have also experienced.<sup>18</sup>

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### Real-time PCR

Although FISH is still considered the gold standard for HER2 amplification in the clinical setting, we sought to study our discordant cases (see below) by a third technique.<sup>19</sup> Real-time quantitative PCR was performed with the LightMix<sup>R</sup> kit HER2/neu (Roche Diagnostics). A 101bp fragment of the HER-2 gene and a 119 bp fragment of the RPL23 reference gene, both localized on chromosome 17q21, were amplified according to the manufacturer's instructions. Simultaneous quantification of the HER2 gene and of the reference gene was accomplished by using two different LightCycler hybridization probes (LightCycler<sup>R</sup> Red 640 and LightCycler<sup>R</sup> Red 670, respectively) enabling dual color detection in a single test tube. A color compensation file generated with the Roche Diagnostics LightCycler<sup>R</sup> Multicolour Compensation Set was used to correct the fluorescence in the duplex reaction. DNA was extracted from FFPE

tumor tissue using a previously described protocol<sup>20</sup> and subsequently amplified in triplicate using a LightCycler 480 real-time PCR instrument (Roche Diagnostics). In each PCR experiment, DNA extracted from tumors with known HER2 amplification status (tumors classified as amplified or non-amplified by both FISH and SISH) was included as positive controls: three samples without amplification and three samples with amplification. Moreover, each PCR experiment included a non-template control and standards (from 10<sup>1</sup> to 10<sup>6</sup> equivalents per reaction of HER2 DNA and of reference DNA) supplied with the kit. These standards allow the generation of standard curve for both products to determine the linear range of both PCR reactions and to estimate the quantity of the target sequence in unknown samples. Briefly, for each reaction 2µl of LightCycler HER2 mix, 2µl LightCycler reference mix, 10µl LightCycler 480 probes master mix and 1µl PCR grade water were combined. 5µl of DNA (for samples and standards) or PCR grade water (for negative control) was added to give a total volume of 20µl. PCR was performed as follows: after an initial ten minutes denaturation of DNA at 95°C, 45 amplification cycles were performed. Each cycle consisted of denaturation at 95°C for ten seconds, annealing at 60°C for ten seconds, and extension at 72°C for ten seconds. The fluorescence signals were measured after each annealing step.

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### Post-analytical Phase (Interpretation)

The slides were analysed by two observers. All the preparation was previously evaluated (10x and 40x objectives in the case of SISH and 100x objective in the case of FISH) to identify areas for scoring and to avoid the bias of tumor heterogeneity.

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For FISH, a total 20 nuclei were scored from two different areas using an epifluorescence microscope (Olympus BX61) equipped with a DAPI, a Spectrum Orange, Spectrum Green and double-filter set, using a 100x oil immersion objective lens. The scoring of SISH was similarly conducted with the use of a bright-field microscope (Olympus BX41) with a 40x objective. Positivity for HER2 gene amplification was considered when ISH ratio was  $\geq 2$ ; negativity when ISH ratio was  $< 2$ . Chromosome 17 polysomy was defined as  $\geq 3$  CEP17 signals on average per cell.<sup>21</sup> Amplification patterns in clusters *versus* double minutes were considered according to published criteria.<sup>22</sup>

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For data analysis in the real-time assay, we used the LightCycler 480 Relative Quantification software (version 1.5) provided by Roche Diagnostics. We used the Second Derivative Maximum method to calculate the value of the crossing point for target and reference genes of each sample. The HER2 copy number was calculated automatically as the ratio between HER2 and the reference gene. The ratio HER2/Reference for each sample was normalized to one of the non-amplified tumors (determined either by FISH or SISH) included as positive controls in the PCR experiment. According to the manufacturer, a ratio between HER2 and the reference gene of  $< 2$  is regarded as negative for HER2 gene amplification, while a ratio of  $\geq 2$  indicates amplification of the HER2 gene.

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### Statistical analysis

The agreement between FISH and SISH was estimated by the percentage of agreement and by kappa statistics. One sample Z test was performed in order

to test the proportion equality for the two histological subtypes (intestinal versus diffuse and indeterminate).

## Results

### FISH

FISH was successfully performed on all samples. The quality of the hybridization was good (Figure 1). 17.5% were amplified, 55% had double minutes amplification and 45% cluster amplification. Heterogeneity (focal amplification) was observed in 52% of the amplified cases. Interobserver agreement was perfect. For one observer, the median of the ratios was 5.25 and the range 13.26. For the other observer, the median of the ratios was 5 and the range 17.9. HER2 amplification was significantly associated with the intestinal histological subtype when compared with the other categories of the Lauren classification (86% versus 14%,  $p < 0.0001$ ).

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### Dual-color SISH

SISH was successfully performed on all samples. The quality of the hybridization was good (Figure 2). 21% were amplified, 34% had double minutes amplification, 46% cluster amplification and 20% mixed amplification pattern. Heterogeneity (focal amplification) was observed in 29% of the amplified cases. Interobserver agreement perfect. For one observer, the median of the ratios was 5.75 and the range 14.64. For the other observer, the median of the ratios was 5.65 and the range 9.01.

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### Correlation between FISH and SISH

In 96.4% of cases FISH showed the same results as SISH. All 6 discordant cases were positive by SISH and negative by FISH (sensitivity=1, specificity=0.956, concordance=0.964, kappa=0.884; Table 1). Upon review of the FISH slides, all contradictory cases were polysomic and were confirmed to be negative for amplification by real-time PCR (see below). Interestingly, all ratios in this latter group (including those of the two observers) were between 2.06 and 2.50 so setting the cut-off for amplification at  $\geq 3$  would result in a perfect concordance (sensitivity=1, specificity=1, concordance=1, kappa=1; Table 2). All the ratios (including those of the two observers) of the SISH concordances were greater than 3 (median=8.1 and 6.02, range=13.66 and 8.02).

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### Real-time PCR

Six tumors with discordant results obtained by FISH and SISH were analyzed by real-time quantitative PCR with the LightMix kit HER2/neu (Roche Diagnostics) in order to classify them as amplified or non-amplified for the HER2 gene (Table 3). Samples amplified by FISH and SISH, considered as a positive control for HER2 gene amplification, were all found to be amplified using the real-time quantitative approach. Similarly, samples assessed as negative for HER2 gene amplification by both FISH and SISH gave a normalized ratio of HER2/RPL23  $\sim 1$  (range from to 0.836 to 1.0), which is below the cut-off limit of two and confirms the absence of amplification. All samples with discordant results show no amplification of the HER2 gene by real-time quantitative PCR. After normalization of the ratio target/reference gene for each

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sample, we found values  $< 2$  for all tumors, confirming the results obtained by FISH.

## Discussion

In the present study we have compared for the first time a novel fully automated dual color SISH with FISH for the assessment of HER2 amplification in a large series of gastric carcinoma samples. There follows discussion of the different phases of the procedures.

Firstly, ISH is not affected by the unavoidable variability of the pre-analytical phase in pathology laboratories worldwide, as long as buffered formalin is used as fixative. In our experience as a referral laboratory for FISH HER2 testing, only 3% were considered non-informative due to pre-analytical aspects (F. López-Ríos, unpublished data). Expanded decalcification protocols even permit ISH in bone marrow biopsies (E. García-García, unpublished data). Although the role of the new rapid fixatives remains to be determined, it must be emphasized that alcohol-based fixation is not appropriate for ISH procedures. In the present series, in spite of studying samples of different sizes (endoscopic versus surgical specimens, etc...) and sources (community hospitals versus large university hospitals, etc...), both methods showed very low failure rates (data not shown). These results are consistent with previous reports on failure rates for EGFR SISH versus FISH.<sup>23</sup>

Secondly, manual ISH (that is, FISH) remains the gold standard in this setting, and will continue to be until proven otherwise. Nevertheless, new automated ISH alternatives may improve the reproducibility of the analytical phase if the technical platforms, reagents and protocols are fully standardized.

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In the past, there were two main limitations to the widespread use of ISH techniques: 1) FISH has traditionally been performed in central or referral laboratories, with lack of community acceptance or experience, and 2) brightfield ISH has not, until recently, been a dual color procedure.<sup>14, 24, 25</sup> The new, fully automated, dual color SISH overcomes both limitations, but appropriate training is essential to perform the post-analytical phase (interpretation) of the procedure adequately. This is somewhat easier and quicker for brightfield ISH than for FISH.<sup>26, 27</sup> It is beyond the scope of this study to establish the predictive value of the different ISH modalities in gastric carcinoma patients who are candidates for HER2 targeted therapies but the use of automated ISH techniques may enable rapid screening of thousands of patients in order to find out the real predictive cut-off for clinical benefit (ie, polysomy, degree of HER2 amplification).

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Discrepancy between the FISH and SISH occurred in six out of 166 cases, all of them positive for SISH (all ratios between 2.06 and 2.50) and negative for FISH. All discrepant cases were polysomic by FISH and negative for HER2 amplification by real-time PCR. These results are consistent with previous studies reporting on both EGFR and HER2 brightfield ISH, both using single color<sup>18, 27, 28, 29, 30, 31, 32</sup> and dual-color<sup>14, 24, 26, 33</sup> approaches. However, it must pointed out that in some series the brightfield ISH results are “false” negatives not “false” positives, a consideration with potential clinical consequences.<sup>14, 24, 33</sup> Interestingly, polysomy has been reported as the major cause of response to trastuzumab in FISH negative breast carcinoma patients.<sup>34</sup> In our series, no cases showing gene amplification by FISH were considered negative by SISH. When we raised the SISH cut-off for amplification

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( $\geq 3$ ), the concordance was 100%, demonstrating that SISH is a valid testing method for HER2 testing in gastric carcinoma patients and that a higher cut-off ( $\geq 3$ ) should be considered even for dual-color SISH in order to increase the concordance rate. It is also necessary to take into account that polysomy may be the cause of interobserver differences.<sup>29</sup> A very recent report using a similar methodology to the one reported herein (fully automated SISH assay with a single color locus detection on two separate slides) has arrived at the same conclusions: chromosome 17 counts are the main source of discrepancy between SISH and FISH ratios.<sup>27</sup> Another potential source of disagreement between ISH techniques is heterogeneity.<sup>23, 24, 28</sup> Although heterogeneity occurred in up to 52% of our amplified cases, it did not cause the discrepancies because, before scoring, screening of the entire slide took place.

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Another interesting aspect to consider is the concordance of ratios between the different ISH techniques. In agreement with some authors<sup>33</sup> and in disagreement with others<sup>25, 26</sup>, there seems to be, in our series, a weak trend toward lower ratios for FISH than for brightfield ISH. This should be taken into consideration if, in the future, the ratio has predictive value, as has been demonstrated in the neoadjuvant setting of breast carcinoma patients.<sup>35</sup> Interestingly, the HERA trial has failed to confirm that the degree of HER2 amplification influences the benefit from adjuvant trastuzumab. However, it is important to emphasize that central FISH analyses were only available in 61% of the patients randomized and that it was performed in two different laboratories.<sup>36</sup> Following that line of reasoning, in the future, large trials could benefit from the availability of robust automated hybridization assays that allow for a rapid (<24 hours), reliable and permanent ISH technique.



An analysis of the literature on ISH in gastric carcinoma is consistent with our results although there is no data yet on the use of dual-color brightfield ISH approaches. A recent publication reviewed this matter. An analysis, combining this information with our literature, shows that, in 2513 samples, the mean HER2 positivity rate was 16.5% (range 6.9-42.6%), very similar to the results of our series. <sup>22, 34, 37, 38, 39</sup> Agreement exists that this alteration is associated with gastric carcinomas of intestinal type but it is controversial whether it is homogeneous or focal.<sup>39, 40</sup> In the present study, HER2 amplification was indeed more frequent in that histologic type and heterogeneous in up to 52% of the samples. This result emphasizes the need to screen the whole slide before scoring, a post-analytical approach that is easier with SISH than with FISH.

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**The implementation of FISH in local laboratories has fallen below the initial expectations (approximately 4% of hospitals with < 300 beds), according to a recent CAP survey.<sup>41</sup> Therefore, it is likely that interpretation of gastric HER2 SISH will be performed by pathologists who are not familiar with FISH. SISH has three main advantages over FISH (permanent record, bright-field post-analytical phase and fully automated analytical phase) that are particularly relevant in gastric HER2 assessment: (1) SISH slides are easier to screen at low power (important with heterogeneity, as mentioned above); (2) co-localization of ISH and IHC findings is straightforward with bright-field assays (approximately 8-13% of GCs may exhibit HER2 genetic-protein discordances); <sup>2</sup> and (3) availability of a fully automated SISH that allow for a rapid subgroup analysis of the ToGA trial samples (mining of this database is of great**

interest to understand why patients with IHC 0 or 1+ and FISH positive do not seem to benefit from trastuzumab).<sup>2</sup>

In summary, we have compared two different ISH approaches for HER2 testing in gastric carcinoma. The excellent concordance and the absence of false negative cases validate this novel automated dual color SISH. The few discrepant results with FISH were caused by polysomy. This shortcoming may be avoided by raising the cut-off for amplification ( $\geq 3$ ). Global and national consensus are urgently needed in this setting as a framework for analytical (technical) and post-analytical (interpretative) training. The implementation of a new methodology should be based on gastric carcinoma data but take into serious consideration the previous highs and lows in the experience of breast cancer HER2 testing.

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#### **Disclosure/conflict of interest**

All authors declare no conflict of interest. This validation study was funded by Roche.

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## Titles and legends to tables

**Table 1:** Performance of silver in situ hybridization (SISH) with a cut-off  $\geq 2$

**Table 2:** Performance of silver in situ hybridization (SISH) with a cut-off  $\geq 3$

**Table 3:** Correlation of *HER2* status studied by FISH, SISH and Real-time quantitative PCR

For Peer Review



## Titles and legends to figures

**Figure 1.** Gastric carcinoma with HER2 amplification. **(a)** Amplification in clusters by FISH and **(b)** SISH. **(c)** Amplification in a double minutes pattern by FISH and **(d)** SISH.

**Figure 2.** *In situ* hybridation of HER2 in gastric carcinoma. **(a)** Non-amplified case by FISH and **(b)** SISH. **(c)** Chromosome 17 polysomy by FISH and **(d)** SISH.

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**Table 1: Performance of silver in situ hybridization (SISH) with a cut-off  $\geq 2$** 

		<i>FISH</i>		<i>Total</i>
		<i>Positive</i>	<i>Negative</i>	
SISH	Positive	29	6	35
	Negative	0	131	131
	Total	29	137	166

**Table 2: Performance of silver in situ hybridization (SISH) with a cut-off  $\geq 3$** 

		<i>FISH</i>		<i>Total</i>
		<i>Positive</i>	<i>Negative</i>	
SISH	Positive	29	0	29
	Negative	0	137	137
	Total	29	137	166

**Table 3: Correlation of *HER2* status studied by FISH, SISH and Real-time quantitative PCR**

<i>Sample Id*</i>	<i>FISH analysis</i>	<i>SISH analysis</i>	<i>Real-time quantitative PCR</i>		
			<i>Ratio HER2/RPL23</i>	<i>Normalized ratio</i>	<i>Status</i>
12	Non-amplified	Non-amplified	0.9272	1.0	Non-amplified
9	Non-amplified	Non-amplified	0.7752	0.8361	Non-amplified
11	Non-amplified	Non-amplified	0.8314	0.8967	Non-amplified
35	Amplified	Amplified	18.70	20.17	Amplified
84	Amplified	Amplified	12.43	13.40	Amplified
85	Amplified	Amplified	1.95	2.103	Amplified
68	Non-amplified	Amplified	0.3276	0.3533	Non-amplified
39	Non-amplified	Amplified	0.8597	0.9271	Non-amplified
67	Non-amplified	Amplified	0.8880	0.9577	Non-amplified
113	Non-amplified	Amplified	0.7460	0.8045	Non-amplified
129	Non-amplified	Amplified	0.5388	0.5811	Non-amplified
8	Non-amplified	Amplified	0.8503	0.9171	Non-amplified

\* Negative controls: samples 12, 9, 11; positive controls: samples 35, 84, 85; discordant cases: samples 68, 39, 67, 113, 129, 8.

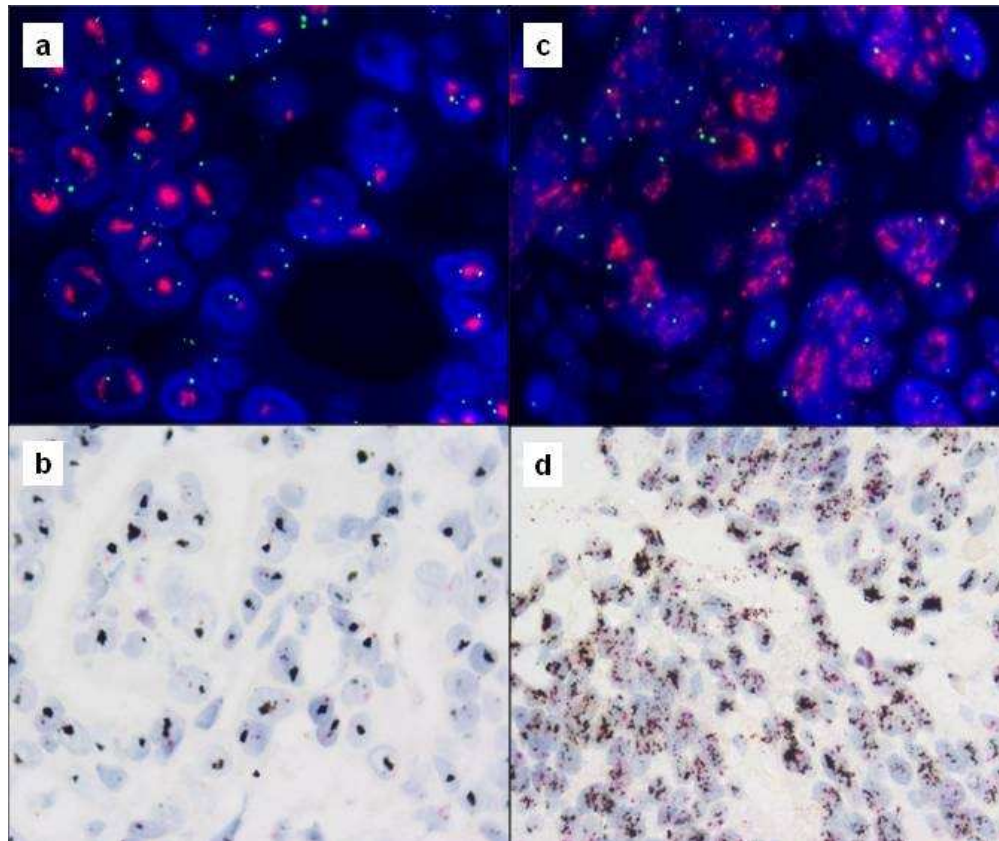


Figure 1. Gastric carcinoma with HER2 amplification. (a) Amplification in clusters by FISH and (b) SISH. (c) Amplification in a double minutes pattern by FISH and (d) SISH.  
177x149mm (96 x 96 DPI)

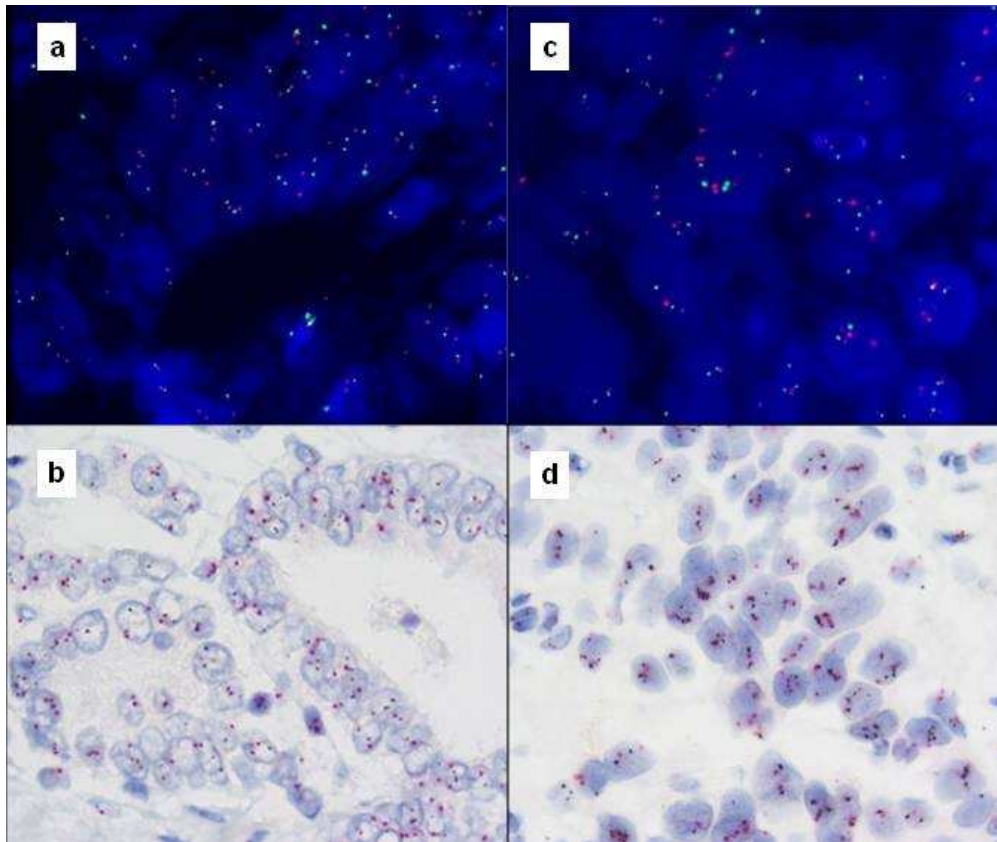


Figure 2. In situ hybridization of HER2 in gastric carcinoma. (a) Non-amplified case by FISH and (b) SISH. (c) Chromosome 17 polysomy by FISH and (d) SISH.  
177x149mm (96 x 96 DPI)