

# Heterogeneity of ERBB2 in gastric carcinomas: a study of tissue microarray and matched primary and metastatic carcinomas

Eun Yoon Cho<sup>1,7</sup>, Kyeongmee Park<sup>2,7</sup>, Ingu Do<sup>3</sup>, Junhun Cho<sup>1</sup>, Jiyun Kim<sup>1</sup>, Jeeyun Lee<sup>4</sup>, Seonwoo Kim<sup>5</sup>, Kyoung-Mee Kim<sup>1</sup>, Tae Sung Sohn<sup>6</sup>, Won Ki Kang<sup>4</sup> and Sung Kim<sup>6</sup>

<sup>1</sup>Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; <sup>2</sup>Department of Pathology, Inje University Sanggye Paik Hospital, Seoul, Korea; <sup>3</sup>Samsung Cancer Research Institute, Sungkyunkwan University School of Medicine, Seoul, Korea; <sup>4</sup>Department of Internal Medicine, Sungkyunkwan University School of Medicine, Seoul, Korea; <sup>5</sup>Biostatistics Department, Samsung Biomedical Research Institute, Samsung Medical Center, Seoul, Korea and <sup>6</sup>Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Trastuzumab in association with systemic cytotoxic chemotherapy is a therapeutic option for patients with advanced or metastatic ERBB2+ gastric carcinoma. The status of the ERBB2 overexpression or gene amplification is an important predictive marker in gastric cancer. However, it is controversial whether the primary tumor is representative of distant metastases in terms of ERBB2 status. Quadruplicated tissue microarrays from formalin-fixed paraffin-embedded tissues from 498 advanced primary gastric carcinomas and 97 matched metastatic lymph nodes were investigated by immunohistochemistry with HercepTest and silver *in situ* hybridization. For further comparison, another set of 41 paired primary and distant metastatic gastric carcinomas were also tested. Intratumoral heterogeneity was defined as different results between tissue microarray cores. ERBB2-positivity was observed in 52 gastric carcinomas (10%) and was not associated with recurrence of disease or survival of patients. In ERBB2-positive primary gastric carcinomas, heterogeneous ERBB2 overexpression was observed in 21/63 (33%) gastric carcinomas and heterogeneous ERBB2 gene amplification in 14/62 (23%) cases. Repeated immunohistochemistry and silver *in situ* hybridization in representative paraffin tumor blocks confirmed focal ERBB2 overexpression and ERBB2 gene amplification and did not change the final results. Discrepancies in ERBB2 results between primary and paired metastatic lymph nodes were observed in 11% of cases by immunohistochemistry and 7% by silver *in situ* hybridization. Out of the 41 paired primary and distant metastases, 5 (12%) cases were ERBB2-positive, and discrepancy was observed in one case. Intratumoral heterogeneity and discrepant ERBB2 results in primary and metastatic tumor are not uncommon in gastric carcinoma. Results of silver *in situ* hybridization showed less frequent heterogeneity compared with immunohistochemistry. Wherever possible, ERBB2 immunohistochemistry testing should be performed in both primary and distant metastatic sites.

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The human protooncogene *ERBB2* (commonly referred to as *HER2*) is a 185-kDa transmembrane tyrosine kinase receptor and a member of the

epidermal growth factor receptor family.<sup>1</sup> Overexpression of ERBB2 has been found to promote tumorigenesis and is involved in the pathogenesis of several human cancers. Amplification of *ERBB2* gene and overexpression of ERBB2 protein in gastric carcinoma were first described in 1986, and since then a number of studies have confirmed these findings.<sup>2</sup>

There is growing evidence that ERBB2 is an important biomarker and key driver of tumorigenesis in gastric cancer, showing amplification or overexpression in 7–53% of tumors.<sup>3–20</sup> A recent

Correspondence: Dr K-M Kim, M.D., Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, #50 Ilwon-Dong, Kangnam-gu, Seoul 135-710, South Korea.  
E-mail: kkmkys@skku.edu

<sup>7</sup>These authors contributed equally to this work.  
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multicenter phase III (ToGA) study showed a significant improvement in overall survival in patients with ERBB2+ advanced gastro-esophageal and gastric adenocarcinoma who were treated with chemotherapy plus trastuzumab compared with patients who received chemotherapy alone.<sup>3</sup> Among various methods for measuring ERBB2 in carcinoma tissue, immunohistochemistry is the most attractive routine test based on issues of cost, convenience, and biological relevance.

Overexpression of ERBB2 protein highly correlates to amplification of the *ERBB2* gene,<sup>21</sup> and a significant discordance of ERBB2 status between the primary carcinoma and metastases has been reported in the breast.<sup>22,23</sup> However, the pattern seen in breast cancer has not yet been fully confirmed in gastric carcinoma.<sup>24</sup> In the stomach, the frequency of immunohistochemistry 0/1+ and fluorescence *in situ* hybridization (FISH)-positive gastric carcinoma is almost as high as that of immunohistochemistry 2+ and FISH-positive samples (23% vs 27%), and ERBB2 expression, rather than *ERBB2* amplification, is significantly correlated with longer overall survival.<sup>3</sup> However, the prognostic significance of ERBB2 in gastric carcinoma is still limited and controversial.<sup>25</sup>

Heterogeneity of target expression within individual cancers and between primary tumors and metastases is an obstacle to determining eligibility for targeted therapy.<sup>20</sup> In gastric carcinoma, ERBB2 heterogeneity has not been studied extensively. Marx *et al*<sup>20</sup> first reported homogeneous (not heterogeneous) amplification and expression of ERBB2 in primary gastric carcinomas and metastatic lymph nodes based on 69 paired one tissue microarray-cored gastric cancer samples. Recently, Kim *et al*<sup>26</sup> suggested that the discordant *ERBB2* amplification observed in metastatic lesions is explained substantially by genetic heterogeneity within primary tumors. Our previous study also showed that focal *ERBB2* gene amplification is more common in gastric carcinomas with no (0 and 1+) or low (2+) ERBB2 expression.<sup>27</sup>

To elucidate the intratumoral heterogeneity in primary and metastatic gastric carcinomas, we investigated overexpression and amplification of ERBB2 in 498 primary gastric carcinomas and 97 matched metastatic lymph nodes and an additional independent 41 paired primary and distant metastatic gastric carcinomas by immunohistochemistry and silver *in situ* hybridization.

## Materials and methods

### Patients

In total, 5133 patients with primary gastric adenocarcinoma underwent surgical treatment at Samsung Medical Center in Seoul, Korea from 2000 to 2004. Among them, 1048 patients with pT3 gastric carcinoma according to the

seventh edition of the International Union Against Cancer classification were reviewed.<sup>28</sup> Cases that underwent palliative surgery or preoperative chemotherapy, had a previous history of gastric surgery, or were diagnosed with multiple gastric cancers or any other malignancies were excluded. We retrieved the formalin-fixed, paraffin-embedded blocks containing the primary tumor with/without metastatic tumors of the lymph nodes from 498 patients, including 95 pN0, 97 pN1, 209 pN2, and 97 pN3 cases. All patients underwent radical gastrectomy with D2 lymph node dissection and adjuvant chemotherapy with 5-fluorouracil and leucovorin and were followed up regularly. The institutional review board approved this retrospective study.

Clinicopathological characteristics obtained from medical records included gender, age, tumor size, location, histological type, overall survival, and disease-free survival. Tumors were classified into two groups based on histology: (1) differentiated, which included papillary, well- or moderately differentiated adenocarcinoma, and well-differentiated mucinous carcinoma; and (2) undifferentiated, which included poorly or undifferentiated adenocarcinoma, signet ring cell carcinoma, and poorly-differentiated mucinous carcinoma. The tumor recurrence patterns were evaluated in patients who underwent R0 resection and were classified as locoregional, peritoneal, distant, or combined recurrence. Clinicopathological variables were analyzed to determine their potential association with ERBB2 overexpression and *ERBB2* gene amplification in gastric carcinoma tissues and metastatic lymph nodes.

To further investigate the discrepancies between primary gastric carcinoma and distant metastasis, an independent cohort consisting of 41 paired primary and distant metastasis specimens were retrieved between September 2007 and February 2012 and were also tested for ERBB2 overexpression and *ERBB2* gene amplification. The sites of distant metastasis included the peritoneum ( $n = 11$ ), liver ( $n = 10$ ), colon ( $n = 6$ ), lung or pleura ( $n = 4$ ), ovary ( $n = 3$ ), bone ( $n = 2$ ), skeletal muscle ( $n = 2$ ), rectal shelf ( $n = 1$ ), larynx ( $n = 1$ ), and axillary lymph node ( $n = 1$ ). For this independent cohort with paired primary and distant metastatic tumors, as biopsy was preferred in most cases, whole-paraffin blocks were used for immunohistochemistry and silver *in situ* hybridization.

### Tissue Microarrays

The surgical specimens were fixed in 10% buffered formalin, processed, and embedded in paraffin using the standard protocol. All hematoxylin and eosin-stained slides were reviewed, and the representative areas were carefully selected and marked on all paraffin blocks. Four tissue cores (0.6 mm diameter) were obtained separately from each primary carcinoma, and two tissue cores were sampled from each metastatic lymph node using an AccuMax (Isu Abxis Co., Ltd.). Four primary gastric carcinoma tissue cores were obtained from a single paraffin block, and a core was taken from each of the subserosal invasive front, both lateral sides, and the

luminal surface area of the tumor. In cases with variable differentiation on hematoxylin and eosin staining, both differentiation areas were selected. For metastatic lymph nodes, 97 pN3 cases were selected, with a mean number of metastatic lymph nodes of 17 (range 7–73), and tissue microarray cores were obtained from separate lymph nodes with massive extent of metastases.

### Immunohistochemistry

ERBB2 protein expression was evaluated using the HercepTest kit (Dako, Glostrup, Denmark) according to the manufacturer's protocol. Tissue sections (3  $\mu$ m) were deparaffinized and rehydrated, and antigens were retrieved for 40 min in citrate buffer (pH 6.1) at 95 °C. DAB was used as the chromogen, and the sections were counterstained with hematoxylin. Three independent pathologists (EC, KP, and KK) with no previous knowledge of clinical or pathological parameters, including silver *in situ* hybridization results, evaluated the staining data. ERBB2 immunoreactivity was scored according to ERBB2 overexpression assessment for biopsy specimens.<sup>3,29,30</sup> In all cases with a 3+ score, the three pathologists agreed perfectly, but in cases with a 2+ score, there was disagreement. The final interpretation was determined by consensus. Intratumoral heterogeneity was defined as different results between tissue microarray cores. In cases with heterogeneity, a representative whole paraffin tumor block was tested to confirm the results.

### Silver *In Situ* Hybridization

Consecutive unstained sections on electrostatically charged slides were prepared from the same tissue microarray paraffin blocks used for hematoxylin and eosin staining and silver *in situ* hybridization and were stained according to the manufacturer's protocols. Sequential *in situ* hybridization procedures for *ERBB2* and CEN17 signal detection were conducted with the INFORM HER2 DNA, and CEN17 probes (Ventana Medical Systems, Tucson, AZ, USA) were performed using the Ventana *ultraView* SISH Detection Kit on a Ventana BenchMark XT automated slide stainer following the instructions provided by the manufacturer. *In situ* hybridization results were interpreted using the new guidelines for gastric ERBB2 by two observers (EC and ID).<sup>31</sup>

### Statistical Analysis

SPSS 15.0 for Windows (SPSS, Chicago, IL, USA) was used to analyze the relationships between clinicopathological characteristics and the observed ERBB2 protein overexpression/*ERBB2* gene amplification. The  $\chi^2$  test and logistic regression analysis were used for univariate and multivariate analyses, respectively. Five-year overall survival was determined and compared between groups using the Kaplan–Meier method and log-rank test. Cox multivariate regression analysis was performed, and 95%

confidence intervals were determined. Results were considered significant when the *P*-values were <0.05.

## Results

### Expression and Amplification of ERBB2 in Primary Gastric Carcinomas

Among 498 primary gastric carcinomas, 52 cases (10%) were immunohistochemistry 3+ or 2+ and silver *in situ* hybridization-positive. ERBB2 overexpression was found in 63/498 (13%) primary gastric carcinomas and 16/97 (17%) metastatic lymph nodes (Figure 1). *ERBB2* gene amplification was observed in 62 (12%) primary gastric carcinomas and 13 (13%) metastatic lymph nodes.

Table 1 summarizes the clinicopathological findings observed in the gastric carcinomas with or without ERBB2 overexpression and *ERBB2* gene amplification. ERBB2 overexpression and amplification was more common in male patients, older patients, Lauren intestinal type, and differentiated histology (*P*<0.05). However, ERBB2-positivity was not associated with size or location of tumor, Borrmann gross type, lymphatic invasion, lymph node metastasis, recurrence of disease, or overall survival of patients (*P*>0.05) (Figure 2). In a subanalysis of intestinal type gastric carcinomas by Lauren (*n* = 175), there was no significant difference in survival between the ERBB2-positive vs -negative groups.

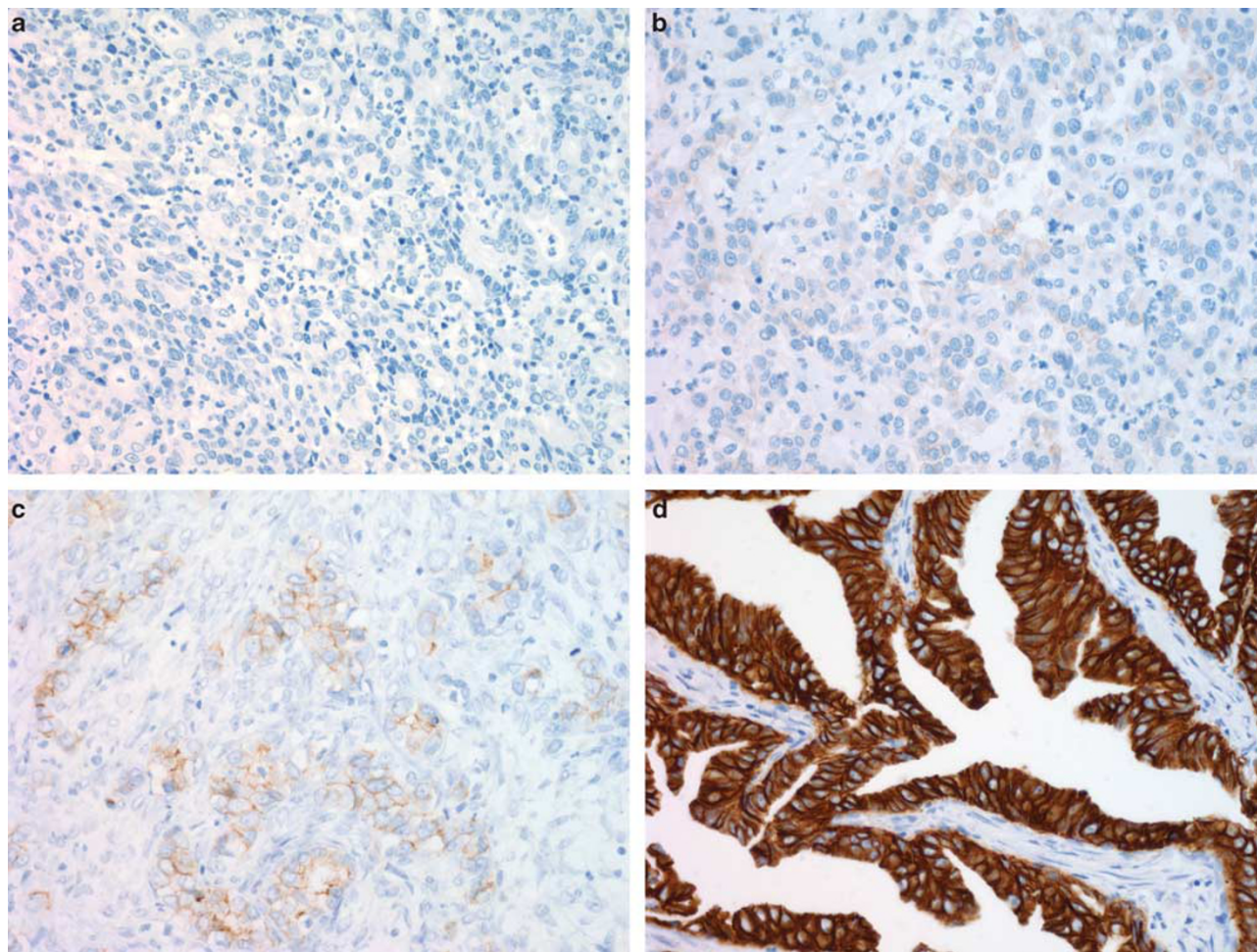
Intratumoral heterogeneity of ERBB2 overexpression in primary gastric carcinoma was observed in 21/63 (33%) gastric carcinomas, and heterogeneous *ERBB2* gene amplification was observed in 14/62 (23%) gastric carcinoma cases (Figure 3). Repeated immunohistochemistry and silver *in situ* hybridization in representative paraffin tumor blocks confirmed focal ERBB2 overexpression and *ERBB2* gene amplification and did not change the final results.

The concordance between immunohistochemistry and silver *in situ* hybridization was 95%, and the correlation between immunohistochemistry and silver *in situ* hybridization results was statistically significant (*P*<0.000001). Among 435 immunohistochemistry 0/1+ cases, 11 (3%) cases showed *ERBB2* gene amplification. Among 21 immunohistochemistry 2+ cases, 10 (48%) cases showed amplification of *ERBB2* gene. All immunohistochemistry 3+ gastric carcinomas showed amplification of *ERBB2* gene.

### Discrepancy of ERBB2 in Primary Tumors and Synchronous Metastatic Lymph Nodes

ERBB2 overexpression was observed in 16/97 (17%) metastatic lymph nodes and 17/97 (18%) corresponding primary gastric carcinomas. *ERBB2* gene amplification was found in 13/97 (13%) metastatic





**Figure 1** In immunohistochemistry, the expression of ERBB2 was interpreted and graded in accordance with the modified system. Scores of (a) 0 or (b) 1+ were considered negative, (c) 2+ was equivocally positive, and (d) 3+ was strongly positive for ERBB2 overexpression.

lymph nodes and 16/97 (17%) corresponding primary gastric carcinomas.

A discrepancy between primary gastric carcinomas and metastatic lymph nodes was noted in 11 (11%) cases by immunohistochemistry and 7 (7%) cases by silver *in situ* hybridization. In those discrepant cases, positive conversion (negative in the primary tumor and positive in the synchronous lymph node metastasis) was observed in 6/81 (7%) cases by immunohistochemistry and 2/81 (3%) cases by silver *in situ* hybridization. Likewise, negative conversion (positive in the primary tumor and negative in the lymph node metastasis) was observed in 5/16 (31%) cases by immunohistochemistry and 5/16 (31%) cases by silver *in situ* hybridization (Table 2).

#### Heterogeneity of ERBB2 in Primary Gastric Carcinomas and Distant Metastases

Of the 41 independent pairs of primary gastric tumors and distant metastases, 5 (12%) primary tumors were ERBB2-positive by immunohistochemistry (4 ERBB2 3+ and 1 ERBB2 2+), and all of

them showed *ERBB2* gene amplification on silver *in situ* hybridization. In paired distant metastases, all ERBB2 3+ cases remained 3+, and one case with 2+ in immunohistochemistry and focal gene amplification of *ERBB2* turned negative in distant metastasis.

#### Discussion

The purpose of this study was to assess intratumoral heterogeneity, discrepancies in ERBB2 status between primary gastric carcinoma and metastatic sites, and the role of ERBB2 in lymph node metastasis and clinical outcomes in patients with advanced gastric carcinoma. A therapeutically significant discordance in ERBB2 status between primary cancer and metastatic sites has been reported in the breast, urinary bladder, and prostate.<sup>23,32,33</sup> These differences could be due to possible genetic drift or clonal selection for ERBB2, which may happen during tumor progression, or to the presence of intratumoral heterogeneity of ERBB2.<sup>23,34</sup> In the present study, a

**Table 1** Patient characteristics according to ERBB2 protein expression and amplification of *ERBB2* gene

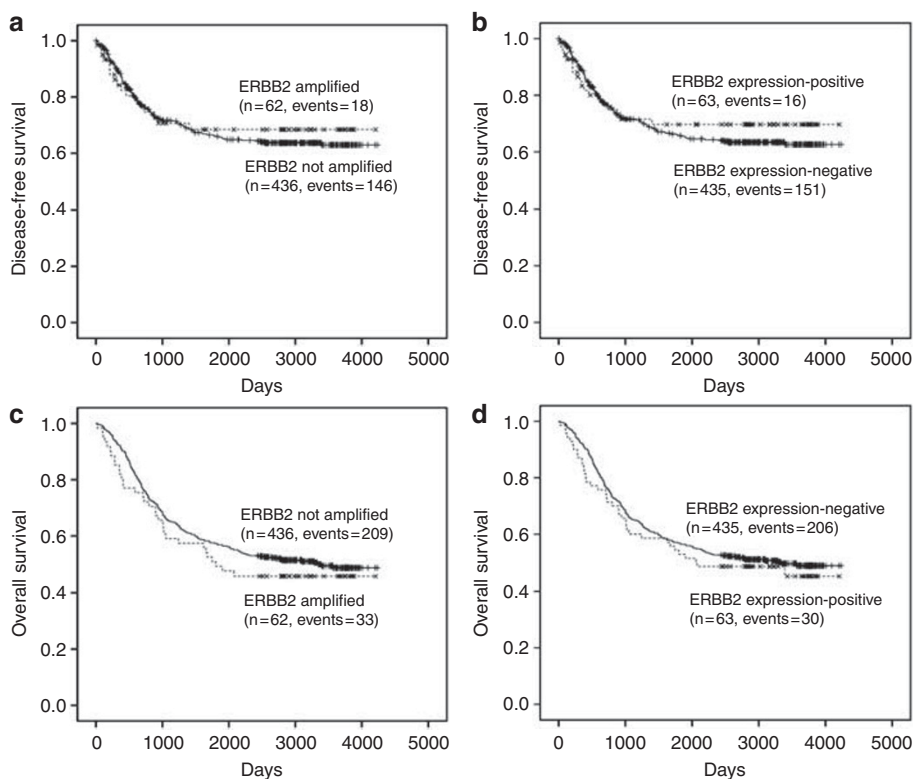
Characteristics, n (%)	ERBB2 immunohistochemistry			ERBB2 SISH		
	Positive (n = 63)	Negative (n = 435)	P-value	Amplified (n = 62) (%)	Not amplified (n = 436) (%)	P-value
<b>Gender</b>			0.046			0.03
Male	49 (78)	279 (64)		49 (79)	279 (64)	
Female	14 (22)	156 (36)		13 (21)	157 (36)	
<b>Age (years)</b>	58.97 ± 11.03	53.53 ± 11.87	0.0007	58.60 ± 11.13	53.6 ± 11.88	0.0019
<b>Tumor location</b>			0.072 (0.85) <sup>a</sup>			0.68 (0.15) <sup>a</sup>
Upper third	9 (14)	70 (16)		10 (16)	69 (16)	
Middle third	17 (27)	183 (42)		19 (31)	181 (42)	
Lower third	32 (51)	153 (35)		29 (47)	156 (36)	
Whole stomach	5 (8)	29 (7)		4 (6)	30 (6)	
<b>Tumor size</b>	6.44 ± 2.42	6.39 ± 3.08	0.91	6.17 ± 2.60	6.43 ± 3.06	0.52
<b>Type of operation</b>			0.81			0.9
Subtotal gastrectomy	39 (62)	261 (60)		37 (60)	263 (60)	
Total gastrectomy	16 (25)	126 (29)		17 (27)	125 (29)	
Extended gastrectomy	8 (13)	48 (11)		8 (13)	48 (11)	
<b>Bormann type</b>			0.32			0.41
IV	5 (8)	58 (13)		5 (8)	55 (13)	
others	58 (92)	380 (87)		57 (92)	381 (87)	
<b>Lauren classification</b>			<0.0001			<0.000001
Diffuse	13 (20)	281 (65)		12 (19)	282 (65)	
Intestinal	47 (75)	128 (29)		47 (76)	128 (29)	
Mixed	3 (5)	26 (6)		3 (5)	26 (6)	
<b>Histology</b>			<0.00001			<0.000001
Differentiated	45 (71)	122 (28)		47 (76)	120 (28)	
Undifferentiated	18 (29)	313 (72)		15 (24)	316 (72)	
<b>Lymphatic invasion</b>			0.89			0.79
Negative	31 (49)	220 (51)		30 (48)	221 (51)	
Positive	32 (51)	215 (49)		32 (52)	215 (49)	
<b>N stage</b>			0.35			0.5
N0	14 (22)	81 (18)		11 (18)	84 (19)	
N1	10 (16)	87 (20)		11 (18)	86 (20)	
N2	23 (37)	190 (44)		24 (39)	189 (43)	
N3	16 (25)	77 (18)		16 (25)	77 (18)	
<b>Recurrence of disease</b>			0.29			0.72
Yes	11 (27)	90 (36)		12 (32)	89 (36)	
No	30 (73)	158 (64)		26 (68)	162 (64)	
<b>Died of disease</b>			1			0.41
Yes	28 (44)	195 (45)		31 (50)	192 (44)	
No	35 (56)	240 (55)		31 (50)	244 (56)	

<sup>a</sup>Comparison between upper third vs other locations.

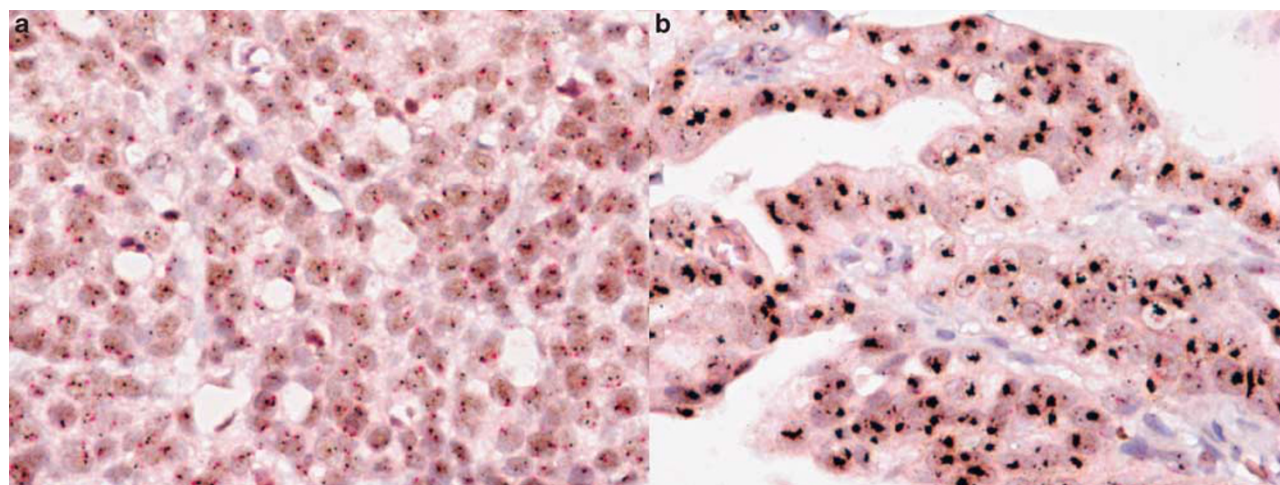
discrepancy in ERBB2 between the primary gastric carcinoma and metastatic lymph node was found in 11% of cases by immunohistochemistry and in 7% by silver *in situ* hybridization. Furthermore, a discrepancy in ERBB2 status between paired primary gastric carcinoma and distant metastasis was also observed in one of the five ERBB2-positive cases. In our study, negative conversion was more frequently observed than positive conversion in metastatic lesions. However, positive conversion was more frequent than negative conversion (2.4 vs

0.4%) in a previous study.<sup>26</sup> In those discrepant cases including ours, heterogeneous *ERBB2* gene amplification was observed in primary tumor. These findings suggest that discordance in ERBB2 status between primary gastric cancer and metastatic sites may be caused by intratumoral heterogeneity of ERBB2 in primary tumor rather than genetic drift during tumor progression. These findings suggest that ERBB2 testing should be performed in both primary and metastatic sites whenever possible for trastuzumab therapy.





**Figure 2** Disease-free survival curves of patients with pT3 gastric adenocarcinoma according to (a) *ERBB2* amplification and (b) *ERBB2* protein overexpression. Overall survival curves of patients with pT3 gastric adenocarcinoma according to (c) *ERBB2* amplification and (d) *ERBB2* overexpression.



**Figure 3** A representative photomicrograph of heterogeneous *ERBB2* gene amplification determined by silver *in situ* hybridization with (a) poorly differentiated adenocarcinoma area without amplification and (b) moderately differentiated adenocarcinoma with amplification.

In previous studies, the overall frequency of *ERBB2* overexpression in gastric cancer ranged from 7 to 53%.<sup>4–20</sup> This wide range of overexpression of *ERBB2* might be explained by the study cohort, localization within the stomach, histological subtype or interpretation of results. *ERBB2* positivity is significantly higher in intestinal histology (34%) and gastro-esophageal tumors (34%) than in diffuse-type (6%) and gastric locations (20%).<sup>25</sup> In the

present study, we used FDA-approved staining procedures and newly developed *ERBB2* scoring criteria for gastric carcinoma, and our *ERBB2*-positivity was similar to recently published large series with 2168 and 506 cases, respectively.<sup>3,35</sup>

The concordance of *ERBB2* protein expression and gene amplification in gastric carcinomas is controversial.<sup>25</sup> *ERBB2* overexpression may occur through a number of different mechanisms,

**Table 2** Comparison of ERBB2 overexpression and ERBB2 gene amplification in primary gastric carcinomas and synchronous metastatic lymph nodes

Metastatic	Primary	ERBB2 immunohistochemistry		Discrepancy	ERBB2 silver <i>in situ</i> hybridization		Discrepancy
		Positive (n = 16)	Negative (n = 81)		Amplified (n = 16)	Not amplified (n = 81)	
<i>ERBB2 immunohistochemistry</i>				11/97 (11%)			
	Positive (n = 17)	11	6		15	6	
	Negative (n = 80)	5	75		1	75	
<i>ERBB2 silver in situ hybridization</i>				7/97 (7%)			
	Amplified (n = 13)	11	2		11	2	
	Not amplified (n = 84)	5	79		5	79	

including transcriptional activation by other genes or post-transcriptional events.<sup>36</sup> Recent studies have reported a high concordance between overexpression in immunohistochemistry and amplification by FISH.<sup>25</sup> In the ToGA trial, the concordance between ERBB2 positivity detected by immunohistochemistry and FISH was 87%, and the differences were largely due to FISH-positive cases that were immunohistochemistry 0/1+, accounting for 22% of all ERBB2-positive cases. In our study, silver *in situ* hybridization-positive and immunohistochemistry 0/1+ cases accounted for 3% of cases, and we achieved 95% concordance between immunohistochemistry and silver *in situ* hybridization.

Marx *et al*<sup>20</sup> reported that *ERBB2* amplification is highly homogeneous in gastric carcinomas. Recently, Kim *et al*<sup>26</sup> reported that the discordant *ERBB2* amplification observed in metastatic lesions is explained substantially by genetic heterogeneity within primary tumors. In our study, we found intratumoral heterogeneity of ERBB2 expression in 4% and gene amplification in 3% of gastric carcinoma cases. In repeated immunohistochemistry and silver *in situ* hybridization performed on whole-paraffin tissue blocks, we found that these heterogeneities were really tumor inherent and not a sampling effect of tissue microarray, and these observations are similar to previous reports.<sup>29,37</sup>

Controversy remains as to whether ERBB2 is a prognostic factor in gastric cancer. Some studies have reported that ERBB2 is a poor prognostic factor in gastric carcinoma.<sup>4–18</sup> However, Grabsch *et al*,<sup>35</sup> Kim *et al*,<sup>37</sup> and Barros-Silva *et al*<sup>38</sup> reported that ERBB2 is not associated with prognosis in large series consisting of 924, 248, and 463 patients, respectively. The disagreement is probably related to differences in sample size, patient characteristics, and interpretation of results.<sup>35</sup> Moreover, most studies have included both early and advanced gastric cancers in their study cohorts, so data concerning exclusively advanced disease are, for the most part, lacking. In this study of 498 patients with advanced

gastric cancer, we found that ERBB2 overexpression or amplification was not associated with lymph node metastasis in pT3 gastric carcinomas and was not a significant prognostic factor.

In summary, intratumoral heterogeneity and discordant ERBB2 results in primary and metastatic tumor are not uncommon in gastric carcinoma. Results of silver *in situ* hybridization showed less frequent heterogeneity compared with immunohistochemistry. Wherever possible, ERBB2 immunohistochemistry testing should be performed in both primary and metastatic sites, and in cases with discrepancy, silver *in situ* hybridization test should be added.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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