Significance of Accurate Human Epidermal Growth Factor Receptor-2 (HER2) Evaluation as a New Biomarker in Gastric Cancer

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Abstract. *Background: HER2 testing in gastric cancer differs from testing in breast cancer because of inherent differences in tumor biology; gastric cancer more frequently shows HER2 heterogeneity and incomplete membrane staining. The aim of the present study was to evaluate the frequency and accuracy of detection of HER2 expression by application of standard criteria in Japanese patients with gastric cancer. Material and Methods: A total of 198 tumor specimens were assessed for HER2 expression by immunohistochemistry (IHC) using the antibodies HercepTest™ and 4B5. Both hand-operated and automated IHC were performed. Results: HER2 expression differed according to the IHC method and antibodies used. HER2 IHC3+ tumors were identified in 21 (10%) and 7 (3.5%) cases by hand-operated and automated IHC, respectively. Conclusion: Among patients with gastric cancer, FISH may be performed in cases of IHC1+ by automated IHC. Further research is required to clarify the relevance of HER2 staining and scoring for the clinical response to HER2-targeted therapy.*

The clinical benefit of trastuzumab in patients with inoperable or metastatic human epidermal growth factor receptor-2 (HER2)-positive advanced gastric or gastroesophageal junction cancer was shown in the Trastuzumab for Gastric Cancer (ToGA) study, an international phase III randomized controlled trial (1). Although many studies have previously evaluated the HER2 status in gastric cancer, the patient cohorts and scoring criteria have varied, resulting in discrepancies in HER2 positivity ranging from 8.2 to 53.4% (2). Frequent heterogeneity of the HER2 status in gastric adenocarcinoma has also made the diagnosis of HER2 overexpression difficult. To solve these problems, the ToGA study employed a new set of immunohistochemistry (IHC) scoring criteria, based on the study by Hofmann *et al*. (3), which consider the biological features of gastric cancer. Using these new criteria, the ToGA study found HER2-positive tumors in 22.1% of metastatic gastric cancer cases. The efficacy of trastuzumab for treating metastatic gastric cancer was clearly demonstrated in the ToGA study, suggesting that anti-HER2 therapy is promising for advanced and metastatic HER2-positive gastric and gastroesophageal cancer. However, the frequency of HER2-positive tumors in patients with resectable gastric cancer, as determined by the new criteria, has not been examined. IHC3+ and fluorescence *in situ* hybridization (FISH)-positive cases were diagnosed as HER2-positive in the ToGA study. FISH-positive cases with an IHC score of 0 were included in the trastuzumab-administered group in the ToGA study, and a difference in overall survival was demonstrated; the FISH-positive group showed a better prognosis. In Japan, the criteria for HER2 positivity in gastric cancer is IHC3+ and FISH-positive IHC2+ cases. Therefore, if patients with an IHC score of 0 or 1 are administered trastuzumab, they may have a better prognosis; however, these patients are not administered trastuzumab in Japan.

To design a proper trial protocol of neoadjuvant or adjuvant therapy using trastuzumab for resectable HER2-positive gastric cancer, the frequency of HER2 positivity in

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Key Words: Human epidermal growth factor receptor-2 (HER2), gastric cancer, immunohistochemistry (IHC).

resectable gastric cancer needs to be determined. Some studies have reported that HER2 expression is associated with a poorer prognosis in gastric cancer (4-6), although a direct correlation has not been proven (7-10). Interpretation of these controversial results is difficult because each study used a different definition of HER2 overexpression or amplification. Regarding the clinicopathological features of HER2-positive gastric cancer, HER2 expression and intestinal histological type have shown a strong correlation (7, 10, 11). When focusing on the IHC staining of the antibody or differentiation of gastric adenocarcinoma, the development of HER2-positive tumors could be linked to the particular type of differentiation. The purpose of this study was to evaluate the frequency of HER2-positive gastric cancer by applying the standard scoring criteria in patients with resected gastric cancer. The relationships between HER2 expression and clinicopathological features and expression rates using several methods were also examined. We, herein, discuss the heterogeneity of HER2 overexpression in gastric cancer, carefully review cases of discordance of HER2 overexpression and gene amplification, and comment on the role of FISH.

Table I. *The relationships between HER2 expression and clinicopathological factors.*

HER2 status Variables	0 (n=101)	1+ (n=48)	2+ (n=28)	3+ (n=21)	*p*-Value
Age	62.4±11.9	62.4±13.3	65.7±13.7	70.8±7.6	N.S.
Gender					
Male	65	28	18	17	
Female	36	20	10	4	N.S.
Lauren's classification					
Intestinal	42	18	15	17	
Diffuse	59	30	13	4	*p*=0.0014
Depth of tumor					
T1,2	68	26	12	10	
T3,4	33	22	16	11	N.S.
pStage					
I	71	29	13	10	
II	7	7	4	4	
III	8	8	5	3	
IV	15	4	6	4	N.S.

IHC: Immunohistochemistry; *HER2*: human epidermal growth factor receptor-2.

Patients and Methods

Patients. Among patients who underwent curative resection for primary gastric cancer at Kyushu University Hospital between January 2003 and December 2007, 198 patients diagnosed with pathological TNM stage I to IV were included in this retrospective study. Clinicopathological parameters, including age, gender, histological classification, and pathological TNM stage, were retrieved from the medical charts or pathology reports. Histological classification was determined according to the Lauren's classification.

Methods. All tissues were fixed with 10% buffered formalin and then paraffin-embedded. Sections (4-μm thick) were de-paraffinized in xylene and hydrated through a graded ethanol series. First, IHC staining of HER2 was manually performed with the HercepTest II™ (DAKO, Glostrup, Denmark). Second, IHC staining of HER2 was performed with the HercepTest II™ and PATHWAY® HER2/neu (4B5) antibodies (Ventana Medical Systems, Tucson, AZ, USA) using an automated slide stainer (BenchMark XT; Ventana Medical Systems). The scoring scheme of the ToGA study was employed for IHC scoring (1) and the results were evaluated by two pathologists (S.A. and Y.Z.).

Out of the 198 specimens, 49 with HER2 defined as either IHC3+ or IHC2+ were evaluated by FISH. FISH analysis was carried-out using the PathVysion HER-2 DNA Probe Kit (Abbott, Des Plaines, IL, USA) after pre-treatment with the Paraffin Pre-treatment Kit (Abbott). Nuclei of invasive tumor cells were scored using a Biozero 8000 microscope (Keyence, Osaka, Japan) equipped with 4',6-diamidino-2-phenylindole (DAPI)/Green/Orange triple bandpass filters. In FISH, the *HER2*/chromosome 17 (Chr17) ratio was determined by counting the *HER2* signals and Chr17 signals in 20 nuclei. Amplification of the *HER2* gene was defined as a *HER2*/Chr17 ratio of >2.2. Negativity for *HER2* amplification was defined as a *HER2*/Chr17 ratio of <1.8. When the ratio was 1.8-2.2, signals in another 20 nuclei were counted, and the *HER2*/Chr17 ratio in a total of 40 nuclei was determined. When the ratio was ≥2.0, amplification was defined as positive; it was otherwise defined as negative.

To compare the stainings, the serial sections of the same block were examined and DNA was gathered from the same part on FISH.

Statistical analysis. Pearson's χ^2 test and Wilcoxon's test were performed to assess the correlation of clinicopathological parameters with HER2 positivity. All *p*-values were two-sided, and $p<0.05$ was considered to be statistically significant.

Results

HER2 positivity and clinicopathological factors. The association of HER2 status with the HercepTest II™ by hand-operated IHC in 198 specimens and the clinicopathological features are summarized in Table I. Out of the 198 patients, 21 (10.6%) and 28 (14%) were diagnosed with HER2 IHC3+ and IHC2+ cancer, respectively; the positive rate was similar to that of the ToGA study. According to Lauren's classification (12), HER2 overexpression was more often detected in the intestinal histological type than in the diffuse type. No correlation was found between HER2-positivity and tumor invasion (pT) or pTNM stage. Table II shows the relationships between HER2 status and several antibodies. HER2 positivity differed according to the IHC method, whether hand-operated or automated. A HER2 IHC3+ status with the HercepTest II™ was present in 21 (10%) specimens by hand-operated IHC. Conversely, a HER2 IHC3+ status was present in 7 (3.5%) and 12 (6%) specimens with the HercepTest II™ and 4B5, respectively, using the automated stainer.

Table II. *Comparison of HER2 expression among several antibodies.*

HER2 status	Hercep test (DAKO)	Automatic	
		Hercep test (DAKO)	4B5 (Ventana)
3+	21 (10)	7 (3.5)	12 (6.0)
2+	28 (14)	9 (4.0)	5 (2.5)
1+	48 (25)	6 (3.0)	7 (3.5)
0	101 (51)	176 (89)	174 (88)

(%)

Table III. *Relationships between HER2 status and FISH results with Hercep test.*

Hercep test (hand-operated)	FISH-positive (n=12)	FISH-negative (n=27)	N.D.
3+ (n=21)	12 (57.1)	6 (28.8)	3 (14.1)
2+ (n=28)	0	21 (66.7)	7 (33.3)

(%)
$p<0.0001$

Diagnosis of HER2 positivity and FISH. The 49 cases shown to be HER2 IHC2+/3+ with the HercepTest II™ were assessed by *HER2* amplification using FISH, and the results are shown in Table III. Of the 21 IHC3+ cases, 12 were FISH-positive, 6 were negative, and 3 were not detected. Of the 28 IHC2+ cases, 21 were FISH-negative and 7 were not detected.

We also examined the relationships between the HER2 status of each antibody and FISH in 39 cases of these, excluding the 10 cases that were not detected. Table IV shows the relationship between the HER2 status and FISH for each antibody. Out of these 49 IHC2+/3+ cases by hand-operated IHC, all 7 cases that were IHC3+ by automated IHC with the HercepTest II™ were FISH-positive (100%). Eleven of the 12 cases that were IHC3+ by automated IHC with 4B5 were FISH-positive (92%). However, some cases became FISH-positive despite the fact that they were negative by automated IHC (cases 30 and 32).

Figures 1 and 2 show the HER2 expression in two cases. Case 26 was HER2-positive by hand-operated IHC and negative by automated IHC. Case 39 was HER2-positive by both IHC methods.

Discussion

The present study included 198 patients with primary gastric cancer, and HER2 expression was assessed using the scoring scheme employed in the ToGA study (1).

Table IV. *Relationships between HER2 status and FISH results with each antibody.*

Case no.	DAKO	DAKO	4B5	FISH
No.1	2	0	0	negative
No.2	2	0	0	negative
No.3	2	0	0	negative
No.4	2	1	1	negative
No.5	2	0	0	negative
No.6	2	0	0	negative
No.7	2	0	0	negative
No.8	2	0	1	negative
No.9	2	0	0	negative
No.10	2	0	0	negative
No.11	2	0	1	negative
No.12	2	0	0	negative
No.13	2	0	0	negative
No.14	2	2	2	negative
No.15	2	1	1	negative
No.16	2	0	0	negative
No.17	2	1	1	negative
No.18	2	0	0	negative
No.19	2	2	2	negative
No.20	2	0	0	negative
No.21	2	0	0	negative
No.22	3	2	2	negative
No.23	3	0	0	negative
No.24	3	2	2	negative
No.25	3	2	3	negative
No.26	**3**	**0**	**0**	**negative**
No.27	3	2	2	negative
No.28	3	3	3	positive
No.29	3	3	3	positive
No.30	3	1	1	positive
No.31	3	3	3	positive
No.32	3	1	3	positive
No.33	3	3	3	positive
No.34	3	3	3	positive
No.35	3	2	3	positive
No.36	3	2	3	positive
No.37	3	2	3	positive
No.38	3	3	3	positive
No.39	**3**	**3**	**3**	**positive**

We performed IHC and FISH to confirm the difference and accuracy of HER2 staining. First, we evaluated the staining difference between the hand-operated and automated stainer. Second, we confirmed whether FISH performed only for HER2 IHC2+/3+ gastric cancer cases is acceptable.

We used the automated stainer BenchMark XT for IHC in the present study; this stainer is used worldwide. This autostaining system has been frequently compared with hand-operated methods. Bankfalvi *et al*. examined the difference between automated and manual determination of the HER2 status in breast cancer (13). Manual

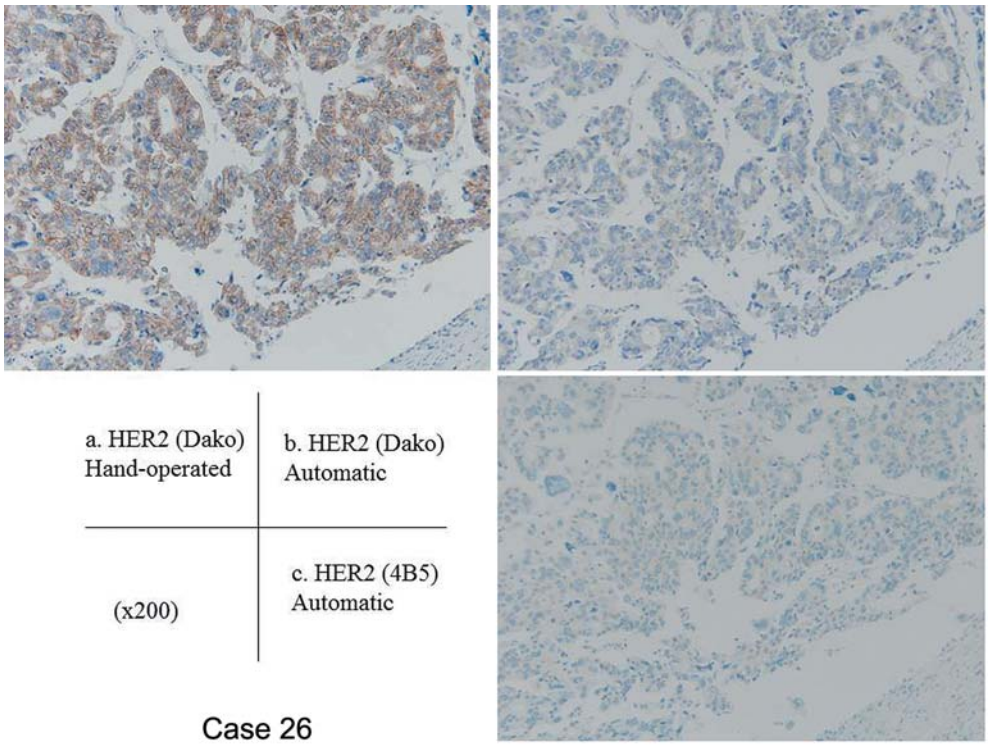

Figure 1. *HER2 IHC staining pattern of case 26. (a) HER2 IHC3+ stained with the HercepTest™ by hand-operated IHC. (b, c) HER2 score of 0 with the HercepTest™ and 4B5 using automated IHC, respectively. Magnification, 400×.*

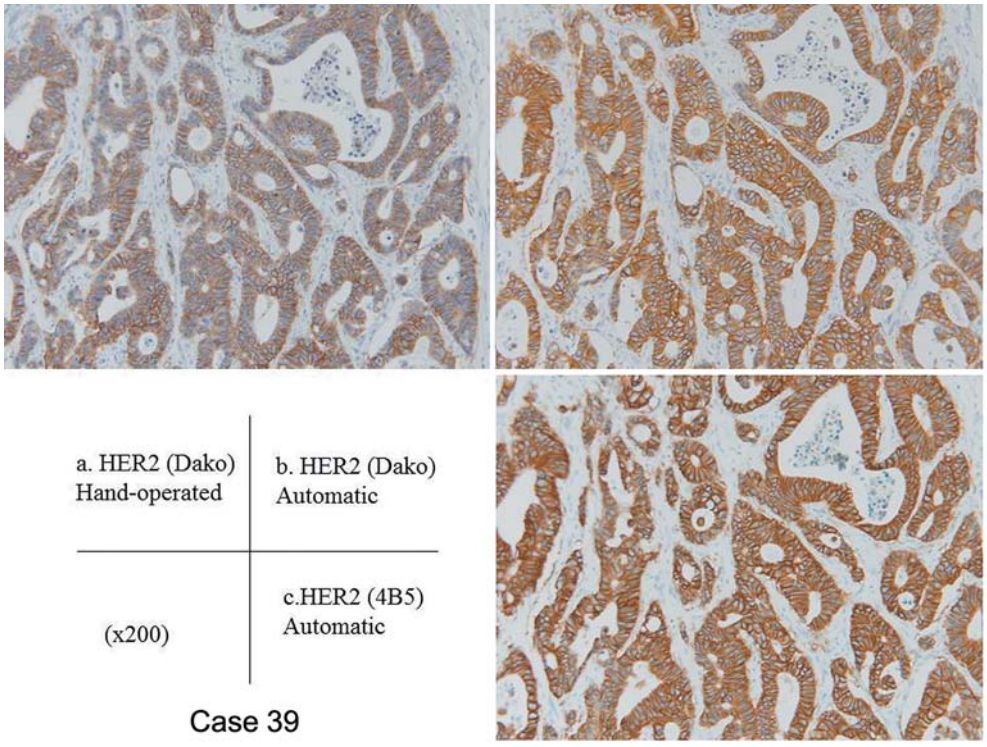

Figure 2. *HER2 IHC staining pattern in case 39. (a) HER2 stained with the HercepTest™ using hand-operated IHC and with (b, c) the HercepTest™ and 4B5 using automated IHC, respectively. All cases were diagnosed as HER2-positive with a score of 3+. Magnification, 400×.*

immunostaining was performed by the HercepTest II™. Automated IHC and FISH were carried-out in the Ventana BenchMark platform using the Pathway-CB11 antibody and the INFORM(R) HER2 probe, respectively. Positivity rates varied between the HercepTest II™ (26%), automated CB11 IHC (23%), and automated FISH (22%) (13). The overall concordance between positive (2+/3+) and negative (0/1+) results of manual and automated IHC was 97%, that between automated FISH and IHC was 92%, and that between automated FISH and the HercepTest II™ was 89%. They concluded that automation improves the accuracy of HER2 detection in diagnostic breast carcinoma tissues (13). By using different methods and different antibodies, the IHC result between antibodies was approximately the same, and it differed from our results.

In the ToGA study, IHC was performed with the HercepTest II™, and FISH was performed with pharmDx (DAKO). The staining method was not described in detail. The estimated HER2-positive ratio was 22.1%, which included IHC 3+ or FISH positivity. In FISH-positive cases, an HER2 IHC 0/1+ status was observed in 61 (10.4%) and 70 (12%) cases, respectively. The present study identified HER2 positivity in 24.7% of all included cases; however, 3-6% of HER2-positive cases were identified with automated IHC. The 49 HER2 IHC2+/3+ cases identified by hand-operated IHC using the HercepTest II™ underwent *HER2* amplification by FISH (Table III). Ten of these 49 cases were proven to be FISH-negative; furthermore, all IHC2+ cases were FISH-negative. The FISH-positive ratio of IHC3+ tumors using automated IHC was higher than that using hand-operated IHC (100% and 92%, respectively). Case 26, whose HER2 status was a score of 3+ by the hand-operated HercepTest II™, scored 0 by automated staining of the two antibodies shown in the serial section in Figure 1. Case 39, whose HER2 status was a score of 3+ by both methods, is shown in Figure 2. Even the same antibody showed different ratios with different staining. The concordance rate between IHC and FISH in the present study was 57.1%, which differs from that of previously published studies (3, 7, 9-11, 14-17); the concordance rate using automated IHC was otherwise similar to that rate. In IHC2+/3+ cases in particular, amplification of the *HER2* gene was confirmed by FISH in all cases. This study also differs from those of breast cancer. HER2 is usually homogeneously expressed in breast cancer; however, HER2 expression in gastric cancer is often heterogeneous (3).

The difference in HER2 expression between the antibodies was lower than that between the IHC methods (hand-operated or automated) in our study. One of the most important reasons for this is the heterogeneous nature of the gastric carcinoma cells in the postoperative specimens. The specimens after curative operation included early gastric cancer; therefore, the amount or areas of carcinoma differed from that of advanced cancer, and heterogeneity was more pronounced.

FISH-positive cases with an IHC score of 0 were included in the trastuzumab-administered group in the ToGA study, in which overall survival improved from 10 to 13 months. It is worth considering that patients with an IHC score of 0 or 1 may benefit from trastuzumab treatment. Based on our results, FISH should be performed for recurrent or unresectable HER2 IHC1+ cases because there were no FISH-negative cases with an IHC score of 0 by automated IHC; however, some IHC1+ cases were FISH-positive. This difference in the HER2-positive ratio between the ToGA study and our study may be attributed to the different backgrounds of patients. The ToGA study only included patients with metastatic or recurrent gastric cancer, while the present study population comprised of patients with resected gastric cancer. A recent study reported an HER2-positive ratio of 8.1% for curatively resected gastric cancer (9), similar to our finding. Taken together, these results suggest that the presence of HER2 positivity might be less frequent in resectable gastric cancer than in metastatic cases.

Of the 21 HER2-positive tumors in the present study, 17 were of the intestinal type according to Lauren's classification. These data are consistent with previous reports in which the intestinal type showed a higher rate of HER2 positivity than the diffuse type (7, 9-11, 14, 16, 17). No correlation was found between HER2 positivity and T- or TNM stage in the present study. HER2-positive tumors were found in 11 of 21 cases of T1/2 cancer (tumor invades as far as the lamina propria or muscularis mucosa). Previous studies that included all pathological stages also reported no correlation between pathological stage and HER2 overexpression (7, 10, 11, 17). Taken together, these findings suggest that HER2 overexpression occurs in the early phase of gastric carcinogenesis. Furthermore, the occurrence of HER2 expression in the early stage strongly suggests that there is no relationship between HER2 expression and prognosis. However, since only small numbers of patients in the early stage were included in these reports, further studies are needed to determine the association between HER2 expression and gastric cancer development. Because HER2 staining results are important in terms of whether patients receive trastuzumab, the accuracy of HER2 staining is important for patients with gastric cancer.

Conclusion

Our study indicated that the HER2-positive rates with hand-operated IHC in patients with resectable gastric cancer were almost identical to that of the ToGA study. A lower HER2 score in resectable gastric cancer has been previously reported; the positive rate of HER2 expression in gastric cancer after curative surgery remains unknown. HER2 expression in gastric cancer differs from that in breast cancer due to heterogeneity. FISH examination should be considered

in cases with an IHC1+ status by automated IHC. Accurate and reliable HER2 testing and scoring will allow for the appropriate selection of patients eligible for treatment with trastuzumab. Further research is required to clarify the relevance of HER2 staining and scoring for the clinical response to HER2-targeted therapy.

Acknowledgements

This research was partially supported by Chugai pharmaceutical CO., Ltd., Japan.

References

1 Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Ruschoff J and Kang YK: Trastuzumab in combination with chemotherapy *versus* chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet *376*: 687-697, 2010.

2 Jorgensen JT: Targeted HER2 treatment in advanced gastric cancer. Oncology *78*: 26-33, 2010.

3 Hofmann M, Stoss O, Shi D, Buttner R, van de Vijver M, Kim W, Ochiai A, Ruschoff J, and Henkel T: Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology *52*: 797-805, 2008.

4 Park DI, Kang MS, Oh SJ, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI, Han WK, Kim H, and Ryu SH, Sepulveda AR: HER-2/neu overexpression is an independent prognostic factor in colorectal cancer. Int J Colorectal Dis *22*: 491-497, 2007.

5 Zhang XL, Yang YS, Xu DP, Qu JH, Guo MZ, Gong Y and Huang J: Comparative study on overexpression of HER2/neu and HER3 in gastric cancer. World J Surg *33*: 2112-2118, 2009.

6 Okines AF, Thompson LC, Cunningham D, Wotherspoon A, Reis-Filho JS, Langley RE, Waddell TS, Noor D, Eltahir Z, Wong R and Stenning S: Effect of HER2 on prognosis and benefit from peri-operative chemotherapy in early oesophago-gastric adenocarcinoma in the MAGIC trial. Ann Oncol *24*: 1253-61, 2013.

7 Marx AH, Tharun L, Muth J, Dancau AM, Simon R, Yekebas E, Kaifi JT, Mirlacher M, Brummendorf TH, Bokemeyer C, Izbicki JR and Sauter G: HER-2 amplification is highly homogenous in gastric cancer. Human Pathol *40*: 769-77, 2009.

8 Grabsch H, Sivakumar S, Gray S, Gabbert HE and Muller W: HER2 expression in gastric cancer: Rare, heterogeneous and of no prognostic value – conclusions from 924 cases of two independent series. Cellular oncology *32*: 57-65, 2010.

9 Kunz PL, Mojtahed A, Fisher GA, Ford JM, Chang DT, Balise RR, Bangs CD, Cherry AM and Pai RK: HER2 expression in gastric and gastroesophageal junction adenocarcinoma in a US population: clinicopathologic analysis with proposed approach to HER2 assessment. Applied immunohistochemistry & molecular morphology: Appl Immunohistochem Mol Morphol *20*: 13-24, 2012.

10 Kim MA, Jung EJ, Lee HS, Lee HE, Jeon YK, Yang HK and Kim WH: Evaluation of HER-2 gene status in gastric carcinoma using immunohistochemistry, fluorescence *in situ* hybridization, and real-time quantitative polymerase chain reaction. Human Pathol *38*: 1386-93, 2007.

11 Yan B, Yau EX, Bte Omar SS, Ong CW, Pang B and Yeoh KG, Salto-Tellez M: A study of HER2 gene amplification and protein expression in gastric cancer. J Clin Pathol *63*: 839-842, 2010.

12 Lauren P: The Two Histological Main Types Of Gastric Carcinoma: Diffuse And So-Called Intestinal-Type Carcinoma. An Attempt At A Histo-Clinical Classification. Acta Pathol Microbiol Scand *64*: 31-49, 1965.

13 Bankfalvi A, Boecker W and Reiner A: Comparison of automated and manual determination of HER2 status in breast cancer for diagnostic use: a comparative methodological study using the Ventana BenchMark automated staining system and manual tests. Int; J Oncol *25*: 929-935, 2004.

14 Yan SY, Hu Y, Fan JG, Tao GQ, Lu YM, Cai X, Yu BH and Du YQ: Clinicopathologic significance of HER-2/neu protein expression and gene amplification in gastric carcinoma. World journal of gastroenterology: WJG *17*: 1501-1506, 2011.

15 Im SA, Kim JW, Kim JS, Kim MA, Jordan B, Pickl M, Han SW, Oh DY, Lee HJ, Kim TY, Kim WH, Yang HK and Bang YJ: Clinicopathologic characteristics of patients with stage III/IV (M(0)) advanced gastric cancer, according to HER2 status assessed by immunohistochemistry and fluorescence *in situ* hybridization. Diagnostic molecular pathology: Amer J Surg Pathol, part B *20*: 94-100, 2011.

16 Boers JE, Meeuwissen H and Methorst N: HER2 status in gastro-oesophageal adenocarcinomas assessed by two rabbit monoclonal antibodies (SP3 and 4B5) and two *in situ* hybridization methods (FISH and SISH). Histopathology *58*: 383-394, 2011.

17 Moelans CB, Milne AN, Morsink FH, Offerhaus GJ and van Diest PJ: Low frequency of HER2 amplification and overexpression in early onset gastric cancer. Cell Oncol (Dordrecht) *34*: 89-95, 2011.

Received April 10, 2014
Revised May 31, 2014
Accepted June 2, 2014