SPANDIDOS PUBLICATIONS

Comparison of *HER2* gene amplification assessed by fluorescence *in situ* hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer

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Abstract. A monoclonal antibody to HER2 protein is widely used in the treatment of patients with HER2-overexpressing breast cancer and has also been found to exhibit antitumor activity in human gastric cancer cells that overexpress HER2. The purpose of this study was to evaluate the frequency of HER2 overexpression and concordance between the results for protein expression and gene amplification in both surgical and biopsy specimens of gastric cancer as assessed with two commercial kits, one for immunohistochemistry (IHC) and the other for fluorescence *in situ* hybridization (FISH). The specimens consisted of formalin-fixed, paraffin-embedded sections of biopsy specimens and surgically resected tumors from 200 cases of invasive gastric cancer that had been treated surgically at the National Cancer Center Hospital East. The lesions were analyzed with the IHC kit, and expression was graded by the United States Food and Drug Administration (FDA)-approved grading system. Gene amplification was evaluated by FISH. IHC revealed HER2 overexpression in 46 of the 200 (23%) cases. The FISH assay was technically successful in 199 cases (99.5%), and gene amplification was observed in 54 cases (27.1%). The concordance rate between the results obtained by IHC and FISH was 86.9%. The concordance rate between the findings in the surgically resected tumors and the 200 pre-treatment biopsy specimens was 88.7%. HER2 expression can be assessed in gastric cancer with a commercial kit as previously reported in breast cancer. Even small biopsy specimens were found to be suitable for evaluating gastric cancer for HER2 overexpression.

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Introduction

The *HER2* (also called c-erbB2) is a proto-oncogene and is located on chromosome 17q21 (1). *HER2* encodes a M_r 185,000 transmembrane glycoprotein, which is a member of the HER receptor family and possesses tyrosine kinase activity. Overexpression of HER2 protein has been described in approximately 25-30% of invasive breast cancers, and it has been used as a marker of resistance to various therapeutic modalities, and short disease-free survival (2,3). Trastuzumab (Herceptin, Genentech, Inc., South San Francisco, CA), a monoclonal antibody to the HER2 protein, is a promising agent for the treatment of breast cancer patients with a poor prognosis, and Slamon *et al* have reported that addition of Trastuzumab to the chemotherapy regimen yields a significantly higher response and prolongs time to progression and overall survival of a breast cancer patients with HER2 overexpression (4).

Various methods are available to determine the HER2 status of breast cancer, however, many of them require fresh tissue, involve a complicated procedure, and are costly. Immunohistochemistry (IHC) is widely used to evaluate protein expression in formalin-fixed, paraffin-embedded specimens, and, Southern blot hybridization is recognized as the standard method for analysis of *HER2* gene amplification, but the procedure requires a large, fresh specimen (5). Fluorescence *in situ* hybridization (FISH) can be used to analyze small formalin-fixed, paraffin-embedded specimens for gene amplification, and Press *et al* have evaluated FISH as a mean of assessing *HER2* amplification in breast cancer (3). In their study, FISH was used to test for *HER2* amplification in 140 breast cancers in which gene amplification had already been demonstrated by Southern hybridization and it was found to have a sensitivity of 98% and a specificity of 100%. IHC and FISH are widely used methods for evaluating HER2 status for breast cancer, furthermore, Food and Drug Administration (FDA) in the United States approved IHC and FISH tests to determine HER2 status for breast cancer patients: Hercep test kit (DakoCytomation Denmark A/S, Glostrup, Denmark), and PathVysion HER2 DNA probe kit (Vysis Inc., Downers Grove, IL). Many investigators have

compared IHC and FISH as means of evaluating HER2 in breast cancer (5-7). In general, correlation level between IHC and FISH results approximately 90% in IHC strong positive cases (3+), however weakly positive cases (2+) obtain only a minor association. Therefore, National Compresive Cancer Network (NCCN) guidelines for treatment of breast cancer recommend that HER2 testing should be done using IHC and/or FISH, an IHC results of 2+ should be confirmed with FISH (8).

Many investigators subsequently evaluated HER2 status in gastric cancer by IHC, and the frequency of HER2 overexpression varied widely, from 8 to 31% (9-14). The consensus of almost all reports is that the majority of positive cases are the intestinal type histologically. Methods of IHC which evaluate for HER2 status in gastric cancer have not been standardized and there is a wide range in the frequency of overexpression, furthermore, there have been few reports claiming to have demonstrated *HER2* gene amplification in gastric cancer (15-17).

In xenograft experiments, Trastuzumab has also shown antitumor activity in gastric cancer cell lines with HER2 overexpression, and synergy has been demonstrated with some cytotoxic agents (18). Results of that study encourage the clinical investigation of Trastuzumab for patients with HER2-overexpressing gastric cancer.

We usually collect small biopsy specimens endoscopically from advanced gastric cancers before initiating chemotherapy. In unresectable cases, tumor behavior before treatment has to be evaluated on the basis of these small specimens alone. However, since gastric cancer is considered to be a heterogeneous tumor, small biopsy specimens may not reflect its behavior.

Before using Trastuzumab in the treatment of gastric cancer, concordance between protein expression and gene amplification of *HER2* has to be confirmed with commercial kits as examined for breast cancer, and the feasibility of using biopsy specimens to evaluate HER2 status must also be confirmed.

The purpose of this study was to evaluate the frequency of HER2 overexpression in gastric cancer and the concordance between protein expression and gene amplification in both surgical and endoscopic biopsy specimens with two commercial kits, an IHC and a FISH.

Materials and methods

A total of 1,254 patients with primary gastric cancer underwent surgery at the National Cancer Center Hospital East (Kashiwa, Japan) between July 1992 and March 2000. Of the 1,254 patients, 261 cases were invasive intestinal-type gastric cancer. We selected 200 of 261 cases in which preoperative endoscopic biopsy samples were completely preserved in our hospital. The specimens consisted of formalin-fixed, paraffin-embedded sections of preoperative endoscopic biopsy specimens and surgically resected tumors.

All tissues were fixed with 10% buffered formalin, generally for 24 and 48 h, and paraffin-embedded. Sections 4-μm thick were cut from a paraffin block of each specimen and applied to slides for IHC, and 5-μm thick sections were cut and applied to slides for FISH.

The IHC analysis was performed with the Hercep test kit (DakoCytomation Denmark A/S) at Dako Cytomation Co. Ltd, Kyoto, Japan. FISH for *HER2* gene amplification was performed with the PathVysion HER2 DNA probe kit (Vysis Inc.) at FALCO Biosystems, Kyoto, Japan.

IHC for HER2 protein expression. The immunohistochemical analysis with the Hercep test was performed according to the manufacturer's guidelines. All reagents were included in the kit. Briefly, heat-induced epitope retrieval was performed on the deparaffinized sections in advance by immersing the slides in Epitope Retrieval Solution (10 mM citrate buffer; pH 6.0), which had been preheated to 95˚C. They were then placed in a 95˚C water bath for 40 min, followed by 20-min at room temperature, then endogenous peroxidase was quenched with Peroxidase Blocking Reagent. Next, the slides were incubated at room temperature for 30 min with ready-to-use rabbit polyclonal antibody to HER2 oncoprotein, and the primary antibody was detected by incubation at room temperature for 30 min with Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat anti-rabbit immunoglobulins). After washing, slides were developed with Substrate Chromogen Solution at room temperature for 10 min. The expression grading and evaluation were performed in accordance with the FDA-approved system for breast cancer (19). Only membrane staining intensity and pattern were evaluated using the 0 to 3+ scale. Scores of 0 or 1+ were considered negative, a score of 2+ was weakly positive when >10% of the tumor cells showed weak to moderate complete membrane staining, and a score of 3+ was strongly positive when a strong complete membrane staining was observed in >10% of the tumor cells (Fig. 1). In the present study, we defined HER2 expression positive when tumor cell staining was evaluated as 2+ or greater with Hercep test.

FISH for HER2 gene amplification. The results of FISH for *HER2* were evaluated using a PathVysion HER2 DNA probe kit which uses a dual-color probe to determine the number of copies of both *HER2/neu* (SpectrumOrange) and CEP17 (chromosome enumeration probe 17) (SpectrumGreen). The kit was used according to the manufacturer's protocol. Briefly, an appropriate formalin-fixed paraffin-embedded tissue block from each case was selected by the pathologists, cut into 5-μm thick sections, and mounted on silane-coated slides (Dako A/S). One of the sections was stained with H&E and used for the microscopic confirmation of the invasive part of the carcinoma tissue, and other sections were used for the FISH assay. The slide for FISH was deparaffinized in Hemo-De for 10 min three times, and then dehydrated in 100% ethanol for 5 min twice. Air-dried tissue sections were treated in 0.2 N hydrochloric acid for 20 min, then washed in distilled water for 3 min. After immersion in Vysis wash buffer for 1 min, they were immersed in pre-treatment solution for 30 min at 80˚C. After washes in distilled water for 3 min and immersion in Vysis wash buffer for 5 min twice, the slides were exposed to protease solution at 37˚C for 20-30 min, then immersed in Vysis wash buffer for 5 min twice, and dried in air for 20 min at room temperature. The slides were subsequently immersed in 10% buffered formalin for 10 min, and then immersed in Vysis wash buffer for 5 min, twice, and dried in air for 15 min

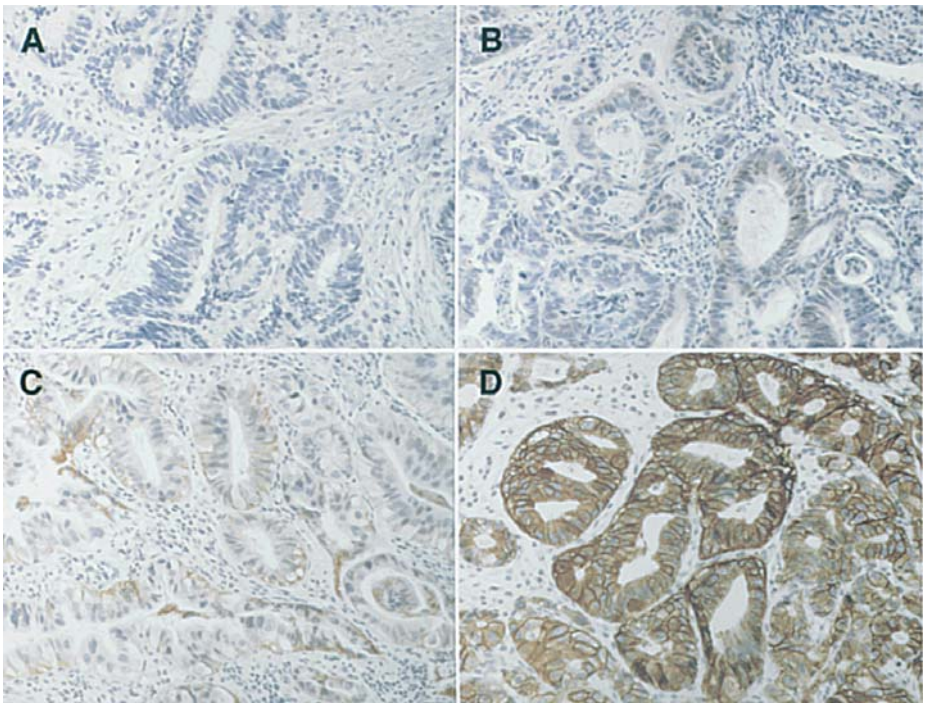

Figure 1. The expression grading and evaluation were estimated in accordance with the FDA-approved system, using the 0 to 3+ scale, score of 0 (A) or 1+ (B) were considered negative, 2+ (C) was weak positive and 3+ (D) was strong positive for HER2 overexpression.

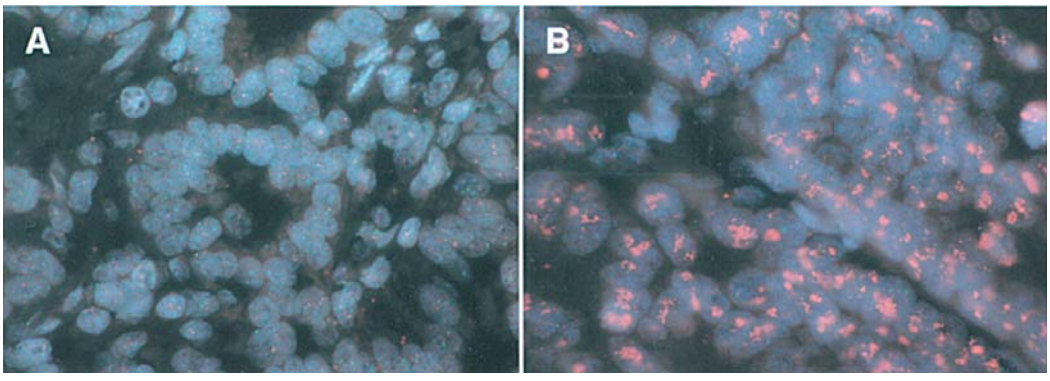

Figure 2. The ratio of *HER2* signals to CEP17 signals was determined, with ratios of <2.0 considered non-amplified (A, negative) and those ≥2.0 amplified (B, positive).

at room temperature. Hybridization was performed at 37˚C for 14-18 h with a denatured DNA probe followed by immersion in pre-warmed post-wash solution at 72±1˚C for 2 min. Finally, the slides were air dried in the dark and counterstained with 4,6-diamidino-2-phenylindole (DAPI).

The total numbers of the *HER2* and CEP17 signals were counted in 60 interphase tumor cell nuclei examined with a fluorescent microscopes and appropriate filters. The ratios of *HER2* signals to CEP17 signals were calculated as follows: when the ratio was <2.0, the gene was considered non-amplified and when it was ≥2.0, the gene was considered to be amplified (Fig. 2).

Statistical analysis. To assess the concordance rate between the results for protein overexpression and gene amplification and between the results for surgically resected tumor and biopsy specimens, we compared the positive and negative rates calculated for each examination. Confidence intervals were computed using the normal approximation to the binomial distribution. Positive predictive value (PPV) was calculated as the number of positive in biopsy specimens divided by the number of positive surgically resected tumors. Negative predictive value (NPV) was calculated as the number of negative biopsy specimens divided by the number of negative surgically resected tumors.

Results

Patient and lesion characteristics are shown in Table I. Median age was 66 years (range 39-88 years), and the male/female ratio was 155/45. There were 194 tubular adenocarcinomas and 6 papillary adenocarcinomas. The pathological stage was: T2/3 in 99/101, N0/1/2/3 in 54/84/47/15, and stage I/II//III/IV in 42/55/67/36 patients.

Table I. Patient and lesion characteristics.

Gender	
Male	155
Female	45
Age	
Median	66
Range	33-88
Histology	
Tubular (tub)	194
Papillary (pap)	6
Pathological TNM classification	
T-stage	
T2	99
T3	101
N-stage	
N0	54
N1	84
N2	47
N3	15
M-stage	
M0	176
M1	24
I	42
II	55
III	67
IV	36

Table II. Her2 overexpression in surgically resected tumors.

IHC score	No. Tub	Pap	Total	%
0	126	0	126	63
1+	28	0	28	14
2+	12	0	12	6
3+	28	6	34	17
Total	193	6	200	
Positive rate (%)	20.6	100		23

Table III. *HER2* gene amplification in surgically resected tumors.

HER2/CEP17[a] ratio	Tub	Pap	Total
Median	1.4	6.8	1.4
Range	1.0-12.3	1.5-9.7	1.0-12.3
<2	144	1	155
≥2	49	5	54
Positive rate (%)	25.3	83.3	27.1

FISH assay was technically successful in 199 (99.7%) of the 200 cases. [a]CEP17, chromosome enumeration probe 17.

HER2 overexpression in surgically resected tumors. The results for HER2 overexpression in the surgically resected tumors are shown in Table II. All 200 cases could be evaluated by IHC. Hercep test score was 0 in 126 cases (63%), 1+ in 28 (14%), 2+ in 12 (6%), and 3+ in 34 cases (17%), respectively. All 6 of papillary adenocarcinomas were 3+ (100%). Of the 200 surgically resected tumor specimens, 46 (23%) of the tumors were found to exhibit HER2 protein overexpression [95% confidence interval (CI): 17-28%].

HER2 gene amplification in surgically resected tumors. The results for *HER2* gene amplification in the surgically resected tumors are shown in Table III. FISH assay was technically successful in 199 (99.7%) of the 200 cases and the *HER2* gene was judged to have been amplified in 54 of 199 cases (27.1%, 95% CI 21-33.2%). The median number of signals per nucleus was 1.4 (range 1.0-12.3) with the median number of signals per nucleus in 54 amplified cases of 5.55. In 194 cases with tubular adenocarcinoma, FISH assay was unsuccessful in 1 case and 49 of the 193 cases (25.3%) were determined to be amplified. Five of the 6 cases (83.3%) of papillary adenocarcinoma were evaluated as amplified with the median number of signals per nucleus of 6.8 (range 1.5-9.7).

Concordance between the results of IHC and FISH in surgically resected tumors. The concordance rate between the results of IHC and FISH in the HER2-protein overexpression cases was 86.7% (58.3% for 2+, 88.2% for 3+) (Table IV). Of the 153 cases that were HER2 protein-negative by IHC, 136 cases were not showed amplification with FISH and its concordance rate was 88.8% (90.4% for 0, 82.1% for 1+). The results of the two assays were concordant in 173 of the 199 cases (86.9%, 95% CI 82.2-91.6%).

Table IV. Concordance between the results of IHC and FISH in surgically resected tumors.

IHC score	FISH Positive	Negative	Concordance rate (%)
0	12	113	90.4
1+	5	23	82.1
2+	7	5	58.3
3+	30	4	88.2
			86.9

Concordance between the results for HER2 overexpression determined by IHC in surgically resected tumors and biopsy specimens. The IHC method was technically successful in all

Concordance between the results for HER2 overexpression determined by IHC in surgically resected tumors and biopsy specimens.

	Surgically resected tumors				
Biopsy specimens	Negative	Positive	Total	NPV (%)	PPV (%)
Negative	144	13	157	91.7	-
Positive	10	33	43	-	76.7
Total	154	46	200		
Concordance rate (%)	93.5	71.7	88.5		

NPV, negative predictive value; PPV, positive predictive value.

of the biopsy specimens (Table V). A total 43 cases were evaluated as positive (21.5%) and 157 cases as negative (78.5%). The concordance rate for HER2 overexpression determined by IHC in the surgically resected tumors and the biopsy specimens was 88.5% (95% CI 87.1-89.9%). Of the 154 cases in which the surgically resected tumor was evaluated as negative for HER2 overexpression by IHC, the biopsy specimen was negative in 144 cases resulted in the concordance rate of 93.5% (95% CI 91-95%). Therefore, 144 of 157 negative cases with biopsy specimens could represent those in surgically resected specimen as HER2 negative (NPV: 91.7%). Of the 46 cases in which surgically resected tumor was evaluated as positive, the biopsy specimens was also positive in 33 cases with the concordance rate of 71.7% (95% CI 58.7-84.7%). Of the 43 cases in which the biopsy specimen was positive, 33 of 43 cases could represent those in surgically resected specimen as HER2 positive (PPV: 76.7%).

Concordance between the results of FISH in surgically resected tumors and biopsy specimens. We have evaluated all 54 biopsy specimens in which gene amplification was demonstrated in the surgically resected tumor: the FISH assay was technically successful in all 54 (100%) cases, despite the small specimens. HER2 was interpreted as being amplified in 33 of these 54 cases, which resulted in the concordance rate of 62.2% (95% CI 49.3-75.1%). The median number of signals per nucleus in the 54 cases was 3.5 (range 1.1-12.2), whereas the median number of signals per nucleus in the 33 amplified cases was 7.9.

Discussion

Gastric cancer is one of the leading causes of cancer death in the world. Despite improvements in survival as a result of early detection and curative surgery, approximately 50,000 patients died of gastric cancer in Japan in 2001 (20). Unresectable advanced cancer and recurrent gastric cancer, in particular, still have a poor prognosis. Randomized trials have demonstrated that fluorouracil (5FU)-based chemotherapy improves survival and quality of life compared with the best supportive care (21-23), however, no standard treatment regimen has been established yet. New and promising agents for gastric cancer are eagerly waited.

Although there is no relevant evidence Trastuzumab has been put forward as a potential candidate in gastric cancer therapy particularly in patients with HER2 expression. However, basic data is needed to decide whether it should be further developed in the treatment for gastric cancer. This is the first large study to evaluate concordance of HER2 status between protein expression and gene amplification in both surgical and endoscopic biopsy specimens of gastric cancer using two commercial kits, an IHC and a FISH.

In this study, HER2 protein overexpression was demonstrated in 23% formalin-fixed paraffin-embedded specimens of surgically resected advanced intestinal type gastric cancers, and *HER2* gene amplification was demonstrated in 27.1%. FISH indicated gene amplification in 86.7% of the cases in which HER2 protein overexpression was detected by IHC, and the concordance rate between the results obtained by IHC and FISH was 86.9%. Takehana *et al* performed a comparative study of IHC and FISH in gastric cancer (17). In their study, IHC revealed HER2 protein overexpression in 29 (8.2%) of 352 surgically resected gastric cancer not only for histologically intestinal type, and FISH showed gene amplification in 25 (86.2%) of the cases with HER2 overexpression. Our results are similar to their analysis and to those analyzed in breast cancer (5-7).

Ridolfi *et al* reported a low frequency of gene amplification in Hercep test 2+ breast cancer cases (24). They reported that FISH demonstrated gene amplification in only 36% of the 2+ cases. They claimed that 2+ IHC reactions are uncertain, that the majority of 2+ cases are a heterogeneous group, and concluded that FISH should be performed on all 2+ cases to confirm gene amplification. Although there have been a few reports of IHC studies of Hercep test 2+ cases, frequency of gene amplification in 2+ breast cancer cases varies widely and is generally lower than in 3+ cases (5-7). According to results of clinical trials of Trastuzumab in breast cancer, its antitumor activity in IHC 2+ cases was consistently lower than in 3+ patients (25,26), therefore Trastuzumab is considered an active agent for breast cancer evaluated as 3+ by Hercep test or as positive for gene amplification by FISH. FISH demonstrated gene amplification in 7 of 12 IHC 2+ cases (58.5%) in the present study, which is clearly lower than in the 3+ cases (88.2%). Taking these results into consideration in the target population for Trastuzumab, FISH should be assessed for patients with IHC 2+ cases even in gastric cancer.

The concordance between the IHC findings in surgically resected tumors and biopsy specimens is very important in gastric cancer clinically. Small specimens of tumors can

easily be obtained endoscopically. If a satisfactory concordance rate is obtained, HER2 status can be evaluated by IHC in unresectable cases as well as cases of recurrence after gastrectomy. In the present study, 21.5% of biopsy specimens were evaluated as positive and the concordance rate between the results in the surgically resected tumors and biopsy specimens was 93.5%. Furthermore, the NPV for the surgically resected tumors was 91.7%, and the PPV was 76.7%. Therefore, we considered it is appropriate to evaluate HER2 status using Hercep test in biopsy specimen for recruiting gastric cancer patients who become candidate for Trastuzumab.

Many investigators have examined that HER2 overexpression could be a predictor of survival outcome in gastric cancer (10-12,14,27-29). Brien *et al* tested 61 cases for gene amplification by performing FISH on sections of paraffin-embedded gastric cancer tissue, and 43% of the cases were positive (28). The multivariate analysis in their study showed that pathological stage and *HER2* gene amplification are independent prognostic factors of survival. Allgayer *et al* confirmed the importance of HER2 status as a prognostic factor in a prospective study of gastric cancer (29). They demonstrated a significant association between level of expression of HER2 and shorter disease-free and overall survival and concluded that HER2 is a promising target for anti-invasive therapy also in gastric cancer. In the present study, there are 36 stage IV patients (18%) who would be target population for systemic chemotherapy. Of the 36 surgically resected tumor specimens, 12 (33%) of the tumors were found to exhibit HER2 protein overexpression, 2+ in 4 (11%), and 3+ in 8 cases (22%), and *HER2* gene amplification were demonstrated in 15 cases (41%). These results might indicate higher tendency of HER2 overexpression and gene amplification in stage IV than in earlier stages, though this should be confirmed in large populations.

HER2 protein overexpression and *HER2* gene amplification can be assessed with commercial kits for breast cancer even in gastric cancer. Even small endoscopic biopsy specimens are suitable for evaluating HER2 overexpression in gastric cancer. Satisfactory concordance rates could be achieved between Hercep test and Pathvision. Development of Trastuzumab for gastric cancer will progress according to the result of this study therefore it could be possible to determine adaptation with Hercep test and PathVysion even in gastric cancer.

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