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ORIGINAL ARTICLE

# HER-2/neu overexpression is an independent prognostic factor in colorectal cancer

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**Abstract**
*Background and aims* The HER-2/neu protein is intimately involved with normal cell proliferation and tissue growth, as it is extensively homologous and is related to the epidermal growth factor receptor. This phenomenon has been most intensively studied in the context of breast carcinoma, in which its amplification and overexpression correlate with the overall course of disease and poor prognoses, and also constitute a predictive factor of poor response to chemotherapy and endocrine therapy. In this study, we investigated the relationships between the expression of HER-2/neu and the clinicopathological characteristics of colorectal cancer, including survival. This study was performed with a view toward the future introduction of Herceptin therapy for colorectal cancer patients.

*Patients and methods* *HER-2/neu* overexpression and gene amplification were examined via semiquantitative standardized immunohistochemical staining and fluorescence in situ hybridization (FISH) in 137 colorectal cancer patients who underwent curative surgery at the Kangbuk Samsung Hospital.
*Results* Sixty-five (47.4%) out of 137 patients were determined by immunohistochemistry to have *overexpressed* HER-2/neu protein. *HER-2/neu* gene amplification was detected in two patients by FISH. Tumors with HER- 2/neu overexpression showed higher postoperative recurrence rate (39.3% vs 14.6%, $p$=0.013). Tumors with HER-2/neu overexpression were associated with poor 3-year (70.8% vs 83.7%) and 5-year survival rates (55.1% vs 78.3%, $p$<0.05). Advanced TNM stage, postoperative recurrence, and overexpression of *HER-2/neu* were found to be independently related to survival by multivariate analysis.
*Conclusion* HER-2/neu overexpression may constitute an independent prognostic factor in colorectal cancer patients, and patients exhibiting HER-2/neu overexpression might constitute potential candidates for a new adjuvant therapy which involves the use of humanized monoclonal antibodies.

**Keywords** Colonic neoplasms · HER-2/neu

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## Introduction

Colorectal cancer is the second most frequent cause of cancer-associated death in the USA [1] and the fourth most frequent cause of cancer-associated death in Korea [2]. Among the prognostic factors now established for colorectal cancer, the most important factor is the TNM stage, which is determined by the depth of invasion, the involvement of the lymph nodes, and distant metastasis. However, prognosis varies among patients in the same

stage, and it is therefore necessary to develop prognostic and predictive factors other than the TNM stage.

The *HER-2/neu* gene is located on chromosome 17q21 and encodes a 185-kDa transmembrane protein which exhibits tyrosine kinase activity [3, 4]. The HER-2/neu protein is extensively homologous and related to the epidermal growth factor receptor (EGFR) [4]. Similar to EGFR, the HER-2/neu protein is involved in normal cell proliferation and tissue growth. Originally, it was observed that transfection of multiple copies of the *HER-2/neu* gene into nonneoplastic human breast cell lines led to increased production of the HER-2/neu protein as well as malignant transformation [5]. HER-2/neu has attracted considerable attention in breast cancer, where it has been targeted successfully in the treatment of patients with advanced disease [6]. In breast cancer, HER-2/neu overexpression has been documented in 10–34% of invasive cancer cases and has been associated with poor prognosis [7]. *HER-2/neu* amplification and overexpression are used as both prognostic and predictive markers for breast cancer. As a prognostic marker, HER-2/neu is used to predict the probable course and outcome of the disease. As a predictive marker, HER-2/neu is used to forecast the patient's therapeutic response to adjuvant chemotherapy and endocrine therapy and to select patients for anti-HER-2/neu monoclonal antibody (Herceptin) immunotherapy. In these patients, treatment with Herceptin has been determined to reduce the volume of tumors, to augment chemotherapeutic effects, and to enhance survival rates in both primary and metastatic breast cancer patients [6, 8].

Favorable clinical results with anti-HER-2/neu antibodies in breast cancer have led to the analysis of HER-2/neu expression in other solid tumors. HER-2/neu amplification and/or overexpression has also been detected in ovarian [9], lung [10], gastric [11], and colon carcinomas [12]. With regard to colorectal carcinomas, several immunohistochemical (IHC) studies have reported different frequencies of HER-2/neu overexpression, in a wide range from 0% to 30% [13–18]. There have been only a limited number of studies that employed fluorescence in situ hybridization (FISH) on *HER-2/neu* gene amplification in colorectal carcinomas, and amplification has been detected in 0–30% of cases [14, 15, 19–23]. There are, however, conflicting results in studies of HER-2/neu with regard to its relationship to prognosis in colorectal cancer patients. Some studies have reported an association between HER-2/neu overexpression and advanced stage, decreased survival, or both [14, 18, 24, 25]. Other studies have failed to find any association with prognosis whatsoever [26–28].

The objectives of this study were (1) to determine the frequency of *HER-2/neu* amplification and overexpression in colorectal cancer, (2) to clarify whether the same mechanisms of gene amplification and protein overexpression function in colorectal cancer as in breast cancer, and (3) to investigate the relationship between *HER-2/neu* amplification/overexpression and the clinicopathological characteristics of tumors, including survival rates. This study was conducted with a view toward the future introduction of Herceptin therapy for the treatment of colorectal cancer patients.

## Materials and methods

### Patients and tissue specimens

A total of 137 colorectal cancer (22 rectal cancer and 115 colon cancer) patients who underwent curative surgery from January 1995 to December 2003 at the Kangbuk Samsung Hospital were included in this study. The experimental group consisted of 75 men and 62 women with ages ranging between 31 and 86 years (mean age 62.4 years). None of the patients had undergone preoperative radiation or chemotherapy. This laboratory study was approved by the Institutional Review Board at the Kangbuk Samsung Hospital, and all patients provided informed consent before being enrolled in the study.

The conditions of these patients were assessed according to the *TNM Classification of Malignant Tumors* [29]. TNM classification revealed that 17 (12.4%) of the patients were in stage I, 47 (34.3%) were in stage II, 60 (43.8%) were in stage III, and 13 (9.5%) were in stage IV.

The causes of death were ascertained from medical records or autopsy, if performed. Patients who had died within 4 weeks of radical surgery were excluded from our analyses. Deaths due to other causes resulted in censored observations beginning at time of death.

Each specimen was routinely fixed in 10% formalin and embedded in paraffin. Before inclusion in the study, each specimen was verified by a histopathologist.

### Immunohistochemical staining

Immunohistochemical staining for HER-2/neu was conducted on 5-μm-thick sections, which were obtained from routine tissue blocks. In brief, after deparaffinization in xylene, the slides were washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by a 15-min incubation in methanol with 3% hydrogen peroxide (Sigma Chemical Co., Deisenhofen, Germany). Nonspecific binding was blocked by the application of normal rabbit serum in a humidity chamber, at a 1:10 dilution, for 30 min. The slides were blotted, and the primary polyclonal rabbit antibody against human HER-2/neu protein (Zymed Laboratories, South San Francisco, CA, USA) was applied for 45 min at room temperature. Secondary goat anti-rabbit antibody (Zymed Laboratories) linked to horseradish peroxidase was applied for 1 h at room temperature. The bound antibody was

visualized using a peroxidase chromogen substrate. The sections were then counterstained with hematoxylin and cover-slipped. The four-tiered scoring system suggested by the manufacturer for use in breast cancer was utilized. Undetectable staining or membrane staining in <10% of the tumor cells was defined as a score of zero. Score 1+ was defined as faint membrane staining in >10% of the tumor cells; 2+ was defined as weak-to-moderate complete membrane staining in >10% of the tumor cells; and 3+ was defined as moderate-to-strong complete membrane staining in >10% of the tumor cells. HER-2/neu protein overexpression was defined as either negative (score 0 and 1+) or positive (score 2+ and 3+). This cutoff point was predicted on the results of previous breast cancer studies. Interpretations were made independently by two pathologists who had been blinded to each other's findings and to the results of the other assays. Control staining was conducted either by omission of the primary antibody, by the use of nonimmune serum and irrelevant antibodies, or by preincubation of primary antibodies with the peptide antigen (1:10; Oncogene Science). We used paraffin slides of invasive breast carcinoma as a positive control.

### Fluorescence in situ hybridization

Fluorescence in situ hybridization analysis was applied to all 2+ and 3+ tumors, as well as to 10 negative tumors (0 and 1+) which were selected at random. We used paraffin slides of invasive breast carcinoma as a positive control. FISH was conducted with the PathVysion *HER-2* DNA Probe Kit (Vysis, Downers Grove, IL) according to the manufacturer's instructions. The slides were evaluated for the *HER-2* gene copy number with an epifluorescence microscope (Zeiss, Thornwood, NY). The PathVysion kit uses two directly labeled fluorescent DNA probes: LSI HER-2, which is specific to the *HER-2* gene locus, and CEP17, which is specific to the alpha satellite DNA sequence at the centromeric region of chromosome 17. Overlapping nuclei were not counted, and split signals were counted as one chromosome component. A cell was considered to show amplification when a definite cluster or more than 10 signals for HER-2/neu were found as in a previous FISH study [30]. Stromal and inflammatory cells were excluded from analysis based on the morphological features of their nuclei.

## Results

The median postoperative follow-up period (minimal–maximal follow-up period) was 48.5 months (1.5–118 months).

### Overexpression of HER-2/neu

Twenty-eight of the 137 investigated colorectal carcinomas were classified as score 0, 44 were classified as score 1+, 38 were classified as score 2+, and 27 were classified as score 3+. Positive immunostaining (2+ or 3+) for the HER-2/neu protein was detected in 65 (47.4%) of the 137 colorectal carcinomas analyzed (Fig. 1). Positive immunos-

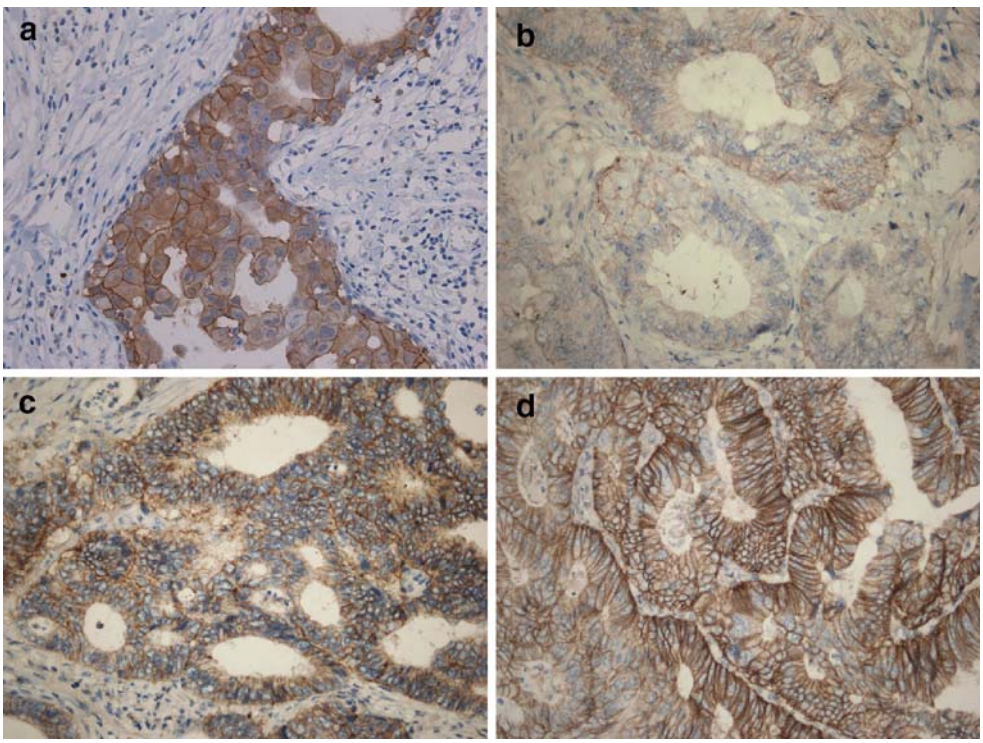


**Fig. 1** Immunohistochemical staining for HER-2/neu (H & E, magnification ×200). **a** Strong complete membrane staining is observed in breast cancer cells (3+, positive control). **b** Faint membrane staining is detected in >10% of tumor cells (1+). **c** Moderate complete membrane staining is observed in >10% of the tumor cells (2+). **d** Strong complete membrane staining is observed in >10% of the tumor cells (3+)

taining (2+ or 3+) was detected in 12 (54.5%) of the 22 rectal carcinomas and 52 (45.2%) of 115 colon carcinomas analyzed ($p>0.05$). Stromal cells and normal epithelial cells adjacent to the tumor tissue were all negative.

The clinical features and pathological data, according to the presence of HER-2/neu overexpression, are summarized in Tables 1 and 2. Tumors with HER-2/neu overexpression showed higher postoperative recurrence rate (39.3% vs 14.6%, $p=0.013$). No relationship was found between HER-2/neu overexpression and age, gender, tumor size, TNM stage, differentiation, lymphovascular invasion, or perineural invasion.

### HER-2/neu amplification

In FISH analysis, gene amplification was detected in two colon carcinomas (Fig. 2). In the 10 tumors evidencing negative immunostaining, none exhibited amplification. When the results of FISH and IHC were compared, among 27 tumors with 3+ immunostaining, two (7.4%) showed amplification. Among the 38 tumors with 2+ immunostaining, none showed amplification. In one tumor, cancer cells had smaller clusters and numerous scattered signals. And in the other tumor, cancer cells had more than 10 homogenous multiple scattered signals without definite signal clusters.

### Survival analysis

Survival analysis was performed on 137 patients who had survived for more than 4 weeks after surgery. The patients with advanced UICC stage showed poor 3-year (stage 1, 90.3%; stage 2, 84.6%; stage 3, 76.3%; stage 4, 63.8%; $p>0.05$) and 5-year survival rates (stage 1, 72.0%; stage 2, 71.4%; stage 3, 68.4%; stage 4, 42.9%; $p>0.05$). The survival curves, according to HER-2/neu overexpression, are shown in Figure 3. Tumors with HER-2/neu overexpression were associated with poor 3-year (70.8% vs 83.7%) and 5-year survival rates (55.1% vs 78.3%, $p<0.05$).

**Table 1** The clinical features according to the presence of HER-2/neu overexpression

| Clinical characteristics | *HER-2/neu* overexpression | | *p* value |
|---|---|---|---|
| | Positive ($n$=65) | Negative ($n$=72) | |
| Age (years, mean±SD) | 64.9±11.0 | 60.6±13.2 | >0.05 |
| Gender (male to female) | 37:28 | 42:30 | >0.05 |
| Chemotherapy | 82.2% | 81.4% | >0.05 |
| Radiotherapy | 35.1% | 28.1% | >0.05 |
| Tumor size | 6.0±1.8 | 6.2±2.5 | >0.05 |
| Location | | | |
| Left/Right-sided | 37/28 | 39/33 | >0.05 |

**Table 2** The pathological features according to the presence of HER-2/neu overexpression

| Pathological characteristics | HER-2/neu overexpression | | *p* value |
|---|---|---|---|
| | Positive ($n$=65) | Negative ($n$=72) | |
| T stage 1/2/3/4 | 8/6/49/2 | 3/5/55/9 | >0.05 |
| N stage 0/1/2/3 | 36/20/9 | 33/24/15 | >0.05 |
| M stage 0/1 | 62/3 | 64/8 | >0.05 |
| TNM stage | | | |
| 1/2/3/4 | 10/24/28/3 | 7/23/32/10 | |
| Differentiation | | | |
| Well/Moderate | 9/54 | 6/59 | >0.05 |
| Poor/Undifferentiated | 1/1 | 7/1 | |
| Lymphovascular | | | |
| Absent/Present | 32/33 | 32/40 | >0.05 |
| Perineural | | | |
| Absent/Present | 64/1 | 69/3 | >0.05 |
| Postoperative recurrence | | | |
| Absent/Present | 39/26 | 61/11 | 0.002 |

### Regression analysis

In our univariate analysis, advanced TNM stage, postoperative recurrence, and HER-2/neu overexpression were all associated with poor survival (Table 3). A Cox proportional hazards model identified advanced TNM stage, postoperative recurrence, and *HER-2/neu* amplification as bearing prognostic importance (Table 4).

## Discussion

Regardless of whether the accumulation of the HER-2/neu is the cause or consequence of carcinogenesis, our results

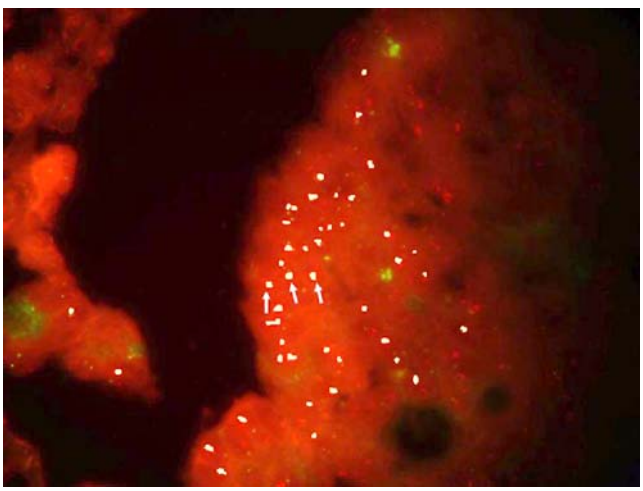

**Fig. 2** Fluorescence in situ hybridization of *HER-2/neu* amplification. Amplified *HER-2/neu* gene forms multiple scattered signals (*white arrows*, magnification ×1000)

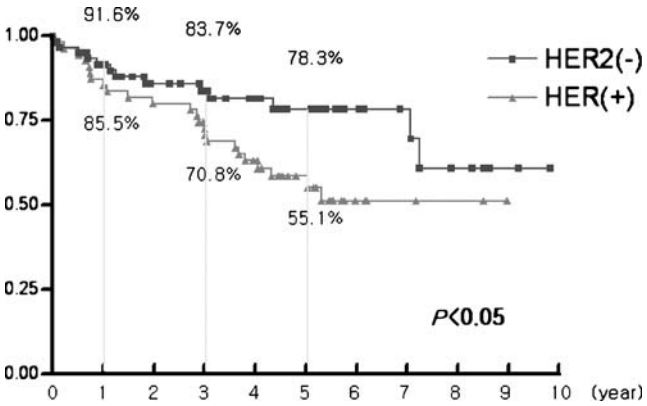


**Fig. 3** Kaplan–Meier plot for overall survival in 137 colon cancer patients according to detection of HER-2/neu overexpression (HER-2/neu positive vs HER-2/neu negative)

clearly indicate that the overexpression of this oncogene correlates with overall survival. Furthermore, regression analysis involving HER-2/neu and the previously established risk factors confirmed that HER-2/neu overexpression is independently related to survival and may constitute a practical tumor marker. Our results are consistent with other prospective and retrospective studies which suggested that HER-2/neu is a prognostic parameter in colorectal cancer.

FISH and IHC are two methods which have been used widely to evaluate HER-2/neu status in clinical laboratories. These methods have both proven sensitive and specific in the detection of either *HER-2/neu* amplification or overexpression using formalin-fixed, paraffin-embedded tissue. Both methods appear to correlate equally well with clinical outcomes in breast cancer patients. Compared to FISH, IHC is less time-consuming, less expensive, requires minimal instrumentation, and is much easier to perform. However, IHC methods can potentially be affected by a host of variables, including tissue fixation, processing, choice of primary antibodies, detection systems, and methods of antigen retrieval [31]. Furthermore, as the suggested scoring system for IHC is subjective, its interpretation may vary among observers. These factors, in addition to small study sample sizes, may also account for the variable rates of HER-2/neu immunoreactivity, as well as the conflicting reports suggesting that HER-2/neu is associated with adverse clinical outcomes in some [14, 18, 24, 25] studies but not others [26–28]. FISH is currently regarded as the "gold standard" for the detection of *HER-2/neu* amplification: it is associated with both high sensitivity (96.5%) and specificity (100%) with regard to the detection of *HER-2/neu* amplification [32]. It also carries the advantage that it can be conducted with small tumor samples and with formalin-fixed and paraffin-embedded tissue samples, with tissue preparation having little or no effect on the testing. It also allows for the direct visualization of gene amplification in the nuclei and provides an objective count of genes and chromosomes on a cell-by-cell basis. However, it also requires a fluorescence microscope and special training in order to interpret the results. It also may prove quite difficult to visualize the morphological features of the tumor cells and also to separate in situ from invasive carcinoma when evaluating the amplification products via fluorescence. In addition, fluorescence fades quickly and therefore does not create a permanent record.

We have found that in the context of colorectal cancer, HER-2/neu overexpression is not frequently associated with gene amplification. This study involved a direct comparison of IHC and FISH in our cases. Among 27 cases exhibiting strongly positive IHC, only 2 (7.4%) evidenced high-level

**Table 3** Survival of 137 patients with colon cancer stratified by clinicopathological features (log rank test)

| | No. of cases | 5-year survival (%) | *p* value |
|---|---|---|---|
| Age (year) | | | |
| <60/≥60 | 51/86 | 68.5/67.6 | 0.9306 |
| Sex | | | |
| Male/Female | 75/62 | 59.0/78.0 | 0.0885 |
| Tumor size | | | |
| <5 cm/≥5 cm | 43/94 | 66.4/68.4 | 0.9684 |
| Histologic grade | | | |
| Well/Moderate/Poor | 12/96/6 | 66.7/71.7/40.0 | 0.1675 |
| TNM stage | | | |
| 1, 2, 3/4 | 17, 47, 60/13 | 72.0, 71.4, 68.4/42.9 | 0.04 |
| Postoperative recurrence | | | |
| Absent/Present | 100/37 | 76.8/53.1 | 0.0017 |
| HER-2/neu overexpression | | | |
| Absent/Present | 72/65 | 78.3/58.6 | 0.0347 |

**Table 4** Multivariate analysis (Cox proportional hazards model)

| | β | SE | RR | 95% CI | *p* value |
|---|---|---|---|---|---|
| Age | 0.02 | 0.02 | 0.30 | 0.98–1.06 | 0.32 |
| Sex | −1.19 | 0.51 | 0.30 | 0.11–0.82 | 0.18 |
| TNM stage | | | | | |
| 1 | | | 1 | | |
| 2 | −1.05 | 0.70 | 0.35 | 0.09–1.38 | 0.13 |
| 3 | 0.388 | 0.56 | 1.47 | 0.49–4.45 | 0.49 |
| 4 | 1.58 | 0.81 | 4.86 | 0.99–23.85 | 0.05 |
| Postoperative recurrence | 1.62 | 0.46 | 5.05 | 2.06–12.41 | <0.001 |
| HER-2/neu overexpression | 1.10 | 0.48 | 2.99 | 1.17–7.64 | <0.001 |

*SE* standard error, *RR* relative risk, *CI* confidence interval

amplification upon FISH, and 25 (92.6%) exhibited no amplification. Consistent with a higher specificity for the gene detection methods, the reported prevalence rates from 10 studies of *HER-2/neu* amplification in colorectal cancer fall into a range (0–30%) somewhat lower than that of the overexpression rates reported for IHC (0–83%). It is generally thought that HER-2/neu overexpression is principally (95%) achieved via gene amplification (increased copies of the normal *HER-2/neu* gene), thereby resulting in increased transcription of the gene, increased HER-2/neu receptors on the cell membrane (overexpression), and increased cell proliferation in breast cancer cases [32]. However, the discrepancy between the expression data and the amplification data shows that gene amplification is not the primary mechanism by which the HER-2/neu protein is overexpressed in colorectal cancer. This is not surprising since HER-2/neu overexpression is known to occur by a number of different mechanisms, including transcriptional activation by other genes or posttranscriptional events [32–38]. Kuwada et al. [39] reported that stable expression of integrin alpha 5/beta 1 in colon cancer cell lines caused a nearly complete loss of HER-2/neu protein via ubiquitination and lysosomal targeting but no change in HER-2/neu mRNA expression compared with control-transfected cells. This discrepancy might also be explained by the fact that colorectal cancer, due to its relatively low rate of HER-2/neu overexpression, is associated with a greater risk for false-positive data due to either nonspecific binding of the primary antibody or the overcalling of low-level binding by a specific antibody. In order to eliminate the possibility of false positivity, however, we counted only complete membrane staining in >10% of the tumor cells as a positive result in our study. For breast cancer, a standardization of the IHC assessments of HER-2/neu overexpression has been introduced, and the concordance rates between these two methods are approximately 73–98%. Concordance between these two methods is particularly high in cases which are completely IHC negative and exhibit no aberrations by FISH, as well as in cases which are strongly IHC positive and show high-level amplification by FISH [40]. Therefore, a standardization of IHC for HER-2/neu in colorectal cancer is clearly warranted.

In summary, our study demonstrates that HER-2/neu overexpression was detected in 47.4% of colorectal cancer patients. Tumors with HER-2/neu overexpression showed higher postoperative recurrence rate. Cox regression analysis verified that advanced TNM stage, postoperative recurrence, and overexpression of HER-2/neu were all independently related to survival. In conclusion, HER-2/neu overexpression may constitute an independent prognostic factor in colorectal cancer patients, and patients with HER-2/neu overexpression may constitute potential candidates for a new adjuvant therapy which involves humanized monoclonal antibodies. Further studies on the role of this oncogene in colorectal cancer will demonstrate whether it can also be used as a target for therapy.

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