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HER2 as a limited predictor of the therapeutic response to neoadjuvant therapy in locally advanced rectal cancer

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ABSTRACT

Human epidermal growth factor 2 (HER2) is a candidate therapeutic and prognostic marker for rectal cancer treated with neoadjuvant chemoradiotherapy. The specific frequency and prognostic role of HER2 protein expression and *HER2* gene amplification in those rectal cancers has not been fully investigated. Pretreatment biopsied and surgically resected formalin-fixed paraffin-embedded tissues from 74 patients were retrospectively evaluated for HER2 protein expression and *HER2* gene copy number using immunohistochemistry (IHC) and silver *in situ* hybridization (SISH), respectively. The tumor response to chemoradiation was evaluated with TNM staging and tumor regression grading (TRG) systems. Good response to chemoradiation therapy (TRG3), poor response (22 TRG1 and 19 TRG2), and TNM downstaging achieved in 33 (44.6%), 41 (55.4%), and 42 (56.8%) patients, respectively. The frequency of HER2 positivity is 17.6%, all of which were low-level *HER2* gene amplification with 2.2 of median gene copy number ratio, detected in IHC0 (3/39), IHC1+ (2/18), IHC2+ (5/14) and IHC3+ (2/3). There was no association of HER2 positivity with clinicopathological parameters or survival. However, older age (≥61 years) and HER2 positivity were the independent predictive factors for non-down staging, while poorly differentiation and the papillary pattern were predictors for poor response. In multivariate analysis, good response proved as an only independent favorable prognostic factor affecting survivals. In conclusion, HER2 positivity may be predictive for a high-risk therapeutic resistance in rectal cancers. The discrepancy between IHC and gene amplification may result from the low-level amplification, which may explain lack of prognostic impact of HER2 positivity.

1. Introduction

Rectal cancer has become one of the leading causes of cancer-related deaths, both in Korea and worldwide [14,33]. Current clinical practice guidelines recommend preoperative chemoradiotherapy followed by radical surgical resection as the standard treatment for locally advanced rectal cancer [4]. Preoperative chemoradiotherapy often downsizes and downstages locally advanced rectal cancers [23], resulting in a complete pathological response in 20.9%–42.2% of patients, with improved overall survival and lower local recurrence rates [9]. The treatment responses and survival outcomes for preoperative chemoradiotherapy are often predicted by the stage, differentiation, and location of the tumor and carcinoembryonic antigen (CEA) levels before treatment [35,38]. However, the tumor can behave in an unexpected manner and these factors do not accurately predict treatment outcomes. Therefore, it is necessary to identify biomarkers that can precisely predict therapeutic response and prognosis to optimize individual treatment. The resected specimen also needs to be examined to determine whether there is a clinically significant risk of treatment failure. There have been many studies attempting to predict the prognosis and therapeutic response based on the features of colorectal cancers [12,17,19,20,24,26,34]. However, those studies have included a variety of tumor sites within the large intestine and have not specifically focused on surgically resected locally advanced rectal cancers treated with preoperative chemoradiotherapy. The clinical significance of mucin pools or papillary tumor cells as a chemoradiotherapy-induced pathological response of the primary tumor in the resected specimens is still unclear. Although mucinous substance in fibrotic areas is not considered as a sign of a vital residual tumor, but rather of therapeutic success. However, it has been recommended that the presence of mucin

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lakes should prompt a search for vital tumor cells or should be treated as a positive result in resection margins [8]. As a result, there is still relatively little known about the potential histopathological factors associated with therapeutic response and prognosis in resected rectal cancers with following neoadjuvant chemoradiotherapy.

The human epidermal growth receptor 2 (*HER2*; *c-ERB-B2*, *HER-2/NEU*) gene, located on chromosome 17q12, is a member of the epidermal growth factor receptor family with tyrosine kinase activity [25]. Receptor activation by dimerization or cleavage of its extracellular domain mediates proliferation signaling via the PI3K/Akt or MAP kinase pathways, resulting in enhanced cell growth, division, and survival [25]. Targeting HER2 using trastuzumab has proven effective in gastric cancer and breast cancer and has led to drug targeting efforts for the treatment of colorectal cancers [3,22]. HER2 protein is expressed in 0.5%–49.6% of colorectal cancers [17,24,26,34], whereas *HER2* gene amplification is observed in 2.5%–18.5% of cancers [24,26,34]. HER2 overexpression has been shown to be associated with poor disease-free survival (DFS) and overall survival (OS) rates in colorectal cancer patients [12,19,20,24]. An *in vitro* study has shown radioresistance in a murine colon cancer cell line overexpressing HER2 [6]. Conversely, neoadjuvant targeted therapy with an anti-HER2 agent has improved the response to radiotherapy of HER2-expressing tumors [6]. Therefore, HER2 expression may be a candidate prognostic or therapeutic indicator for rectal cancer patients being treated with neoadjuvant chemoradiotherapy. However, it remains unclear whether HER2 expression confers radioresistance clinically in rectal cancers [7,12,24,30,37]. The specific frequency and prognostic role of HER2 protein expression and *HER2* gene amplification exclusively in rectal cancers has not been fully investigated [30]. In addition, there have been few studies examining *HER2* gene amplification patterns, the relationship between protein overexpression and gene amplification of HER2, and HER2 expression changes between pre- and post-treatment specimens in rectal cancers. These studies are needed to provide rationale for the use of targeted therapies for rectal cancer patients.

The purpose of this study was to investigate the prevalence of HER2 overexpression, its amplification pattern, its relationship with expression, and the clinically relevant role of HER2 positivity as a therapeutic or prognostic marker for locally advanced rectal cancer patients receiving neoadjuvant chemoradiotherapy and surgery.

2. Materials and methods

2.1. Patients

A retrospective analysis was carried out on data from 113 consecutive patients who received preoperative chemoradiotherapy after a diagnosis of rectal adenocarcinoma at Hallym University Sacred Heart Hospital, Anyang, Korea, between July 2002 and December 2014. Subjects were included in the study if they had a complete tumor resection, with no macroscopic or microscopic residual tumor tissue after total mesorectal excision and if both preoperative and postoperative specimens were available. Subjects were excluded if they had evidence of distant metastasis, familial adenomatous polyposis, multiple colorectal tumors, or synchronous malignancy with colorectal cancers. Cases showing complete tumor regression after chemoradiotherapy and those for which postoperative specimens were unavailable for HER2 analysis were also excluded. In addition, seven patients who had transferred to other hospitals after conducting preoperative chemoradiotherapy, six patients who had not received surgery because of the aggravation of disease or refusal of operation, and one patient who had a lack of data evaluating surgical tissues were excluded. Although perioperative demographic clinicopathological variables for the remaining 99 patients were available, both preoperative and postoperative specimens were only available in 74 patients. Finally, those 74 patients were enrolled in the study. Patients underwent radiotherapy in the pelvic field, with a dose of 45.0–50.4 Gy in 28 fractions over 5 weeks and received concurrent intravenous 5-fluorouracil (5-FU)-based chemotherapy. All patients underwent complete surgical resection, including low anterior resection (n = 46, 62.1%), coloanal anastomosis (n = 11, 14.9%), and abdominoperineal resection (n = 17, 23.0%) 6 to 8 weeks after completion of chemoradiation. Demographic and other variables were retrieved from medical records and tumor registry data. Clinical information, including age, gender, location of tumors, preoperative CEA level, tumor recurrence, and cancer-related death during follow-up, were analyzed. The clinical stages, including depth of invasion (cT), lymph node metastasis (cN), and distant metastasis (cM) were preoperatively evaluated by CT and/or MRI. Cases were histopathologically reviewed and both the clinical and pathological TNM classifications were determined according to the 7th edition of the American Joint Committee on Cancer classification guidelines. The study was approved by the institutional review board of Hallym University Sacred Heart hospital (IRB 2016-1090).

2.2. Pathological examination

Histological slides were reviewed and evaluated by a gastrointestinal pathologist (M.J.K). Biopsy tissues obtained before chemoradiation from 74 patients who underwent colonoscopy were compared with post-treatment surgical specimens to assess the differences in pre- and post-chemoradiation HER2 status. Pretreatment biopsies of the study cases were examined for histological type and tumor differentiation. Based on a hematoxylin and eosin-stained slide review, the available formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks were selected for immunohistochemistry (IHC) and silver *in situ* hybridization (SISH).

The resected specimens after neoadjuvant therapy were also histopathologically investigated for specimen size, tumor regression grade (TRG), lymph node status, post-neoadjuvant treatment TNM stage (ypTNM), the presence of mucinous components in the tumor, and the presence of any papillary pattern of papillary projections with a fibrovascular core within the tumor [31] (Fig. 1A, B). Stromal mucin associated with neoplastic cells was considered to be a mucinous component.

The response of the primary tumor to chemoradiotherapy was assessed using the 5-point TRG system of Dworak et al. [8], as follows: Grade 0, no regression; Grade 1, minor regression (fibrosis in ≤ 25% of the tumor mass, Fig. 1C); Grade 2, moderate regression (dominant tumor mass with obvious fibrosis in 26–50% of the tumor mass); Grade 3, good regression (dominant fibrosis outgrowing the tumor mass with > 50% tumor regression); Grade 4, total regression (only a fibrotic mass, with no viable tumor cells). Grades of TRG3 and TRG4 were defined as a good response and TRG1 and TRG2 were defined as a poor response.

2.3. HER2 protein expression by IHC and HER2 gene amplification by SISH

All 74 specimens were evaluated before chemoradiation and after surgical resection by both IHC and SISH. IHC staining was performed with 4 μm-thick FFPE sections, using a BenchMark XT automated tissue staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA) with validated protocols [20]. Endogenous peroxidase activity was blocked using H_2O_2 before incubating the sections with specific antibodies. Before the application of each primary antibody, sections were treated with ethylenediaminetetraacetic acid and boric acid in Tris buffer (CC1 reagent; Ventana Medical Systems) to aid antigen retrieval. Samples were then incubated with a primary anti-HER2 (1:1000; Dako, Glostrup, Denmark) or anti-EGFR antibody (pre-diluted; Ventana Medical Systems) for 40 min at 37 °C, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (Universal HRP Multimer; Ventana Medical Systems) for 8 min at 37 °C. Tissue sections were then incubated with the chromogen, diaminobenzidine (ultraView Universal DAB Detection Kit, Ventana Medical Systems) and were counterstained with hematoxylin.

IHC was scored according to the consensus panel recommendations on HER2 scoring for gastric cancer [28], This scoring method has previously been used for rectal cancers [15,24]. HER2 staining was scored by counting the number of cells with positive membrane staining

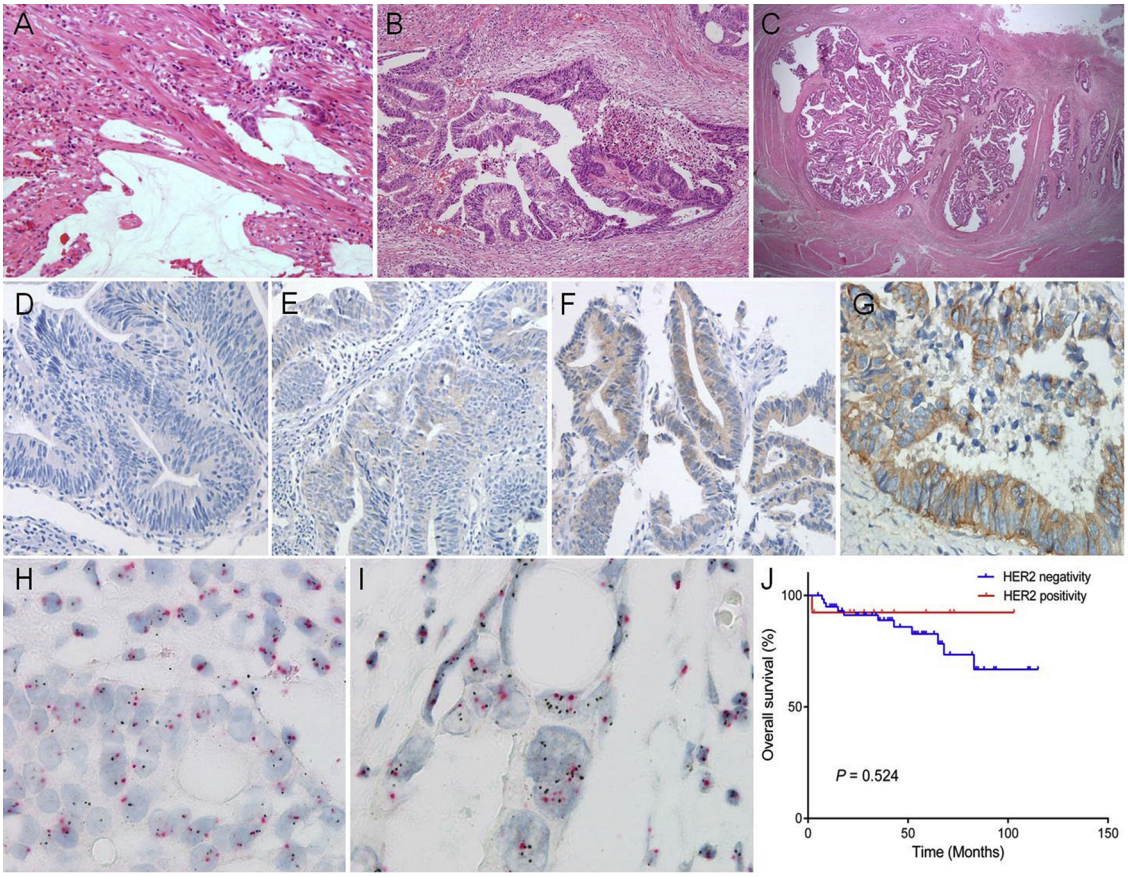

Fig. 1. Histologic features of extracellular mucin (A) and papillary pattern (B) in pretreatment biopsy specimens tend to be related with poor therapeutic response (TRG 1) (C). HER2 protein expression by immunohistochemistry; score 0 (D), score 1 (E), score 2 (F), score 3 (G). (H) Negative for *HER2* gene amplification assessment by silver *in situ* hybridization. Tumor cells display normal disomy with two black (HER2 probe) and two red (chromosome 17 probe) signals. (I) Positive for *HER2* gene amplification. Tumor cells display low-level *HER2* gene amplification with 2–14 black (HER2 probe) signals and 2–7 red (chromosome 17 probe) signals. (J) Kaplan-Meier survival analysis shows no significant association between HER2 positivity and overall survival ($P = 0.524$).

and was expressed as a percentage of the total number of tumor cells as follows: 0, no staining or membrane staining in < 10% of tumor cells; 1+, faint/barely perceptible membrane staining in ≥ 10% of tumor cells; 2+, weak-to-moderate, complete basolateral or lateral membrane staining in ≥ 10% of tumor cells; and 3+, strong, complete basolateral or lateral membrane staining in ≥ 10% of tumor cells.

SISH staining was performed with an automated slide stainer (Ventana Medical Systems) on 4 μm-thick FFPE sections, according to the manufacturer's protocols [16]. SISH was chosen because it can be used with bright field microscopy, which is an advantage due to the heterogeneous nature of HER2 overexpression/amplification in gastrointestinal cancers, especially gastric cancers. Additionally, SISH slides can be stored for a longer period of time without bleaching than FISH slides. Black (HER2) and red (CEP17) signals were quantified in at least 20 non-overlapping tumor cells. Single signal, minor signal clusters, and major signal clusters were counted as 1, 6, and 12, respectively. The total HER2 score was divided by the total *CEP17* score and the HER2/CEP17 ratio was calculated. A HER2/CEP17 ratio ≥ 2.0 was defined as positive for amplification, while a HER2/CEP17 ratio < 2.0 was defined as negative for amplification [15]. Additionally, tumors harboring an average copy number of ≥ 10.0 signals/cell or a HER2/CEP17 ratio ≥ 5.0 were considered to have high-level amplification. Polysomy was defined as the presence of ≥ 3 copies of *CEP17* per cell [15]. An IHC score of 3+ for *HER2* gene amplification was considered HER2-positive, and an IHC score of 0 or 1+ was considered HER2-negative. In samples where the score was 2+, SISH was performed to confirm HER2 positivity [36,38].

2.4. Statistical analyses

A Chi-square test or two-tailed Fisher's exact test was used to compare possible associations of HER2 positivity, tumor response to chemoradiation, and downstaging with categorical clinicopathological variables. Kappa (κ) statistics were used to evaluate the agreement of pre- and post-treatment HER2 protein expression data. κ-values were divided into the following scales to evaluate the strength of the agreement: $\kappa < 0.00$, poor; $0.00 < \kappa < 0.20$, slight; $0.21 < \kappa < 0.40$, fair; $0.41 < \kappa < 0.60$, moderate; $0.61 < \kappa < 0.80$, substantial; and $0.81 < k < 1.00$, nearly perfect.

Survival analysis was performed using the Kaplan-Meier method, with a log-rank test. OS was defined as the interval from the first day of chemoradiotherapy until death or the end of the follow-up period. DFS was defined as the interval from the first day of chemoradiotherapy until tumor progression, death, or the end of the follow-up period. The Cox proportional hazards model was used for multivariate analyses of DFS and OS rates. OS and DFS were analyzed until June 2016. The Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of patients

Patient demographics and tumor characteristics are summarized in Table 1. The median age of patients in this study was 61 years (range,

Table 1
Baseline patient and tumor characteristics.

Demographics	No. of patients (%)
Gender	
Male	52 (70.3)
Female	22 (29.7)
Age (y), median age (range)	61 (29–92)
≥ 61	37 (50.0)
< 61	37 (50.0)
Location of primary tumor	
AV ≥ 6cm	36 (48.6)
AV < 6cm	38 (51.4)
Surgery	
Low anterior resection	46 (62.2)
Coloanal anastomosis	11 (14.9)
Abdomino-perineal resection	17 (22.9)
CEA level at diagnosis	
CEA ≥ 5 ng/mL	33 (44.6)
CEA < 5 ng/mL	41 (55.4)
Clinical T category	
cT2	6 (8.1)
cT3	63 (85.1)
cT4	5 (6.8)
Clinical N category	
cN0	20 (27.0)
cN+	54 (73.0)
Clinical stage	
II	20 (27.0)
III	54 (73.0)
ypT category	
ypT1	5 (6.8)
ypT2	15 (20.2)
ypT3	49 (66.2)
ypT4	5 (6.8)
ypN category	
ypN0	52 (70.3)
ypN+	22 (29.7)
TNM staging change after neoadjuvant treatment	
Downstaging	42 (56.8)
Non-downstaging	32 (43.2)
Tumor regression grading	
TRG 1	22 (29.7)
TRG 2	19 (25.7)
TRG 3	33 (44.6)
Follow-up periods (months), median (range)	40 (2 – 115)
Recurrences during follow-up	18 (24.3)
Death during follow-up	12 (16.2)

AV, anal verge; CEA, carcinoembryonic antigen; c, clinical tumor or nodal staging on CT/MRI before treatment; p, pathologic staging after surgery; TRG, tumor regression grading.

29–92 years) and the majority of patients were male (70.3%, male/female: 52/22). The median distance of the tumor from the anal verge was 5.7 cm (range 1–13 cm). At the time of diagnosis, tumors located ≥ 6 cm from the anal verge occurred in 36 patients (48.6%) and elevated serum CEA levels (≥ 5 ng/mL) were detected in 33 (44.6%) patients. Preoperatively, cT3 was the most common clinical stage (n = 63, 85.1%), followed by cT2 (n = 6, 8.1%) and cT4 (n = 5, 6.8%). Twenty patients (27.0%) were lymph node-negative (cN0), while 54 (73.0%) showed positive lymph nodes (cN+). Most patients (73.0%) had clinical stage III rectal carcinoma.

Histopathological examination of surgically resected specimens after chemoradiotherapy revealed a response grade of TRG1 in 22 (29.7%) patients, TRG2 in 19 (25.7%) patients, and TRG3 in 33 (44.6%) patients. Therefore, a good response to chemoradiation therapy (TRG 3) was achieved in 33 (44.6%) patients and a poor response (TRG1–2) in 41 (55.4%) patients. Comparing the clinical stage to the pathological stage after chemoradiation (ypTNM staging), TNM downstaging was achieved in 42 patients (56.8%). During a median follow-up period of 40 months, 18 patients (24.3%) presented with recurrence, including 13 with systemic recurrence, 3 with locoregional recurrence, and 2 with both systemic and locoregional recurrence. Twelve patients (16.2%) died due to disease progression.

3.2. Determination of HER2 positivity and its clinicopathological correlations

HER2 status was investigated using IHC and SISH in 74 biopsied specimens before chemoradiation. In pre-treatment specimens, HER2 protein expression was scored as 0 in 39 cases (52.7%), 1+ in 18 cases (24.3%), 2+ in 14 cases (18.9%), and 3+ in 3 cases (4.1%, Fig. 1D–G). The overall mean *HER2* gene copy number ratio of rectal cancers was 1.28 ± 0.40 (range, 1.00–2.40), whereas it was 1.16 ± 0.37 for samples with a HER2 staining grade of IHC 0, 1.37 ± 0.39 for IHC 1+, 1.43 ± 0.41 for IHC 2+, and 1.50 ± 0.62 for IHC 3+. *HER2* non-amplification was found in 62 cases (83.8%, Fig. 1H). The median *HER2* gene copy number ratio was 2.2 (range, 2.1–2.4), which was classified as low-level amplification (Fig. 1I). Polysomy was identified in 27 cases (36.5%), 9 of which displayed *HER2* amplification. None of cases showed any clusters of black spots in the analysis of *HER2* gene amplification. *HER2* gene amplification was observed in 12 cases (16.2%). It was detected in 7.7% of cases graded as IHC 0 (3/39), 11.1% of IHC 1+ cases (2/18), 35.7% of IHC 2+ cases (5/14), and 66.7% of IHC 3+ cases (2/3, Table 2). Despite low HER2 expression (IHC 0 or 1+), three samples graded as IHC 0 and two samples graded as IHC 1+ showed *HER2* gene amplification. In contrast, despite high HER2 expression (IHC 3+) in one case (33.3%), there was no *HER2* amplification. Considering that the survival benefit associated with trastuzumab is greatest in IHC 3+ or IHC 2+ and SISH- or FISH-positive patients, 13 (17.6%) cases with a score of 3+ or *HER2* gene amplification were finally considered as HER2-positive.

We further investigated the changes in HER2 protein expression between pre- and post-treatment specimens in 74 patients, to examine the effect of chemoradiation on HER2 status. In 51 cases (68.9%), the HER2 IHC scores of pre-treatment samples corresponded to the scores of post-treatment samples (κ coefficient = 0.519). This association was highly significant statistically ($P < 0.001$).

Table 2
Comparison of HER2 status between immunohistochemistry and silver *in situ* hybridization in pretreatment and post-treatment samples.

		HER2 IHC score (Pre-Tx)			
HER2 SISH	Total N = 74 (%)	0 n = 39 (%)	1+ n = 18 (%)	2+ n = 14 (%)	3+ n = 3 (%)
Amplification	12 (16.2)	3 (7.7)	2 (11.1)	5 (35.7)	2 (66.7)
Non-amplification	62 (83.8)	36 (92.3)	16 (88.9)	9 (64.3)	1 (33.3)
HER2 IHC (Post-Tx)					
0	35 (47.3)	28 (71.8)	5 (27.8)	2 (14.3)	0 (0.0)
1+	20 (27.0)	9 (23.1)	10 (55.6)	1 (7.1)	0 (0.0)
2+	14 (18.9)	2 (5.1)	2 (11.1)	10 (71.5)	0 (0.0)
3+	5 (6.8)	0 (0.0)	1 (5.5)	1 (7.1)	3 (100)

IHC, immunohistochemistry; Pre-Tx, pretreatment; SISH, silver *in situ* hybridization; Post-Tx, posttreatment.

Table 3
Correlations between clinicopathological parameters and HER2 positivity in 74 patients with rectal cancers.

	Total	HER2		*P*-value
	n = 74	Positive n = 13 (%)	Negative n = 61 (%)	
Age (years)				0.543
≥61	37 (50.0)	5 (38.5)	32 (52.5)	
< 61	37 (50.0)	8 (61.5)	29 (47.5)	
Gender				0.928
Male	52 (70.3)	9 (69.2)	43 (70.5)	
Female	22 (29.7)	4 (30.8)	18 (29.5)	
Tumor site				0.545
AV ≥ 6cm	36 (48.6)	5 (38.5)	31 (50.8)	
AV < 6cm	38 (51.4)	8 (61.5)	30 (49.2)	
CEA level				0.762
CEA ≥ 5 ng/mL	33 (44.6)	5 (38.5)	28 (45.9)	
CEA < 5 ng/mL	41 (55.4)	8 (61.5)	33 (54.1)	
Tumor size				0.551
≥5cm	18 (24.3)	4 (30.8)	14 (23.0)	
< 5cm	56 (75.7)	9 (69.2)	47 (77.0)	
Differentiation				1.000
Well/ Moderate	66 (89.2)	12 (92.3)	54 (88.5)	
Poor	8 (10.8)	1 (7.7)	7 (11.5)	
Mucin				0.928
Present	22 (29.7)	4 (30.8)	18 (29.5)	
Absent	52 (70.3)	9 (69.2)	43 (70.5)	
Papillary pattern				0.063
Present	19 (25.7)	6 (46.2)	13 (21.3)	
Absent	55 (74.3)	7 (53.8)	48 (78.7)	
ypT				0.493
ypT0-pT2	20 (27.0)	2 (15.4)	18 (29.5)	
ypT3-pT4	54 (72.0)	11 (84.6)	43 (70.5)	
ypN				0.154
ypN0	52 (70.3)	7 (53.8)	45 (73.8)	
ypN1-2	22 (29.7)	6 (46.2)	16 (26.2)	
Tumor regression grade				0.901
TRG 1-2	33 (44.6)	6 (46.2)	27 (44.3)	
TRG 3	41 (55.4)	7 (53.8)	34 (55.7)	
TNM staging				0.217
Down-staging	42 (56.8)	5 (38.5)	37 (60.7)	
Non-downstaging	32 (43.2)	8 (61.5)	24 (39.3)	

AV, anal verge; CEA, carcinoembryonic antigen; p, pathologic staging after surgery; TRG, tumor regression grading. *Statistically significant, $P < 0.05$.

Correlations between HER2 positivity and clinicopathological parameters of patients are depicted in Table 3. HER2 positivity was not associated with any clinical parameters, including age ($P = 0.543$), gender ($P = 0.928$), tumor location ($P = 0.545$), or CEA levels ($P = 0.762$). Likewise, there were no associations between HER2 positivity and any pathological parameters, including tumor size ($P = 0.551$), tumor differentiation ($P = 1.000$), the presence of extracellular mucin ($P = 0.928$), or papillary pattern ($P = 0.063$). In addition, HER2 positivity was not associated with ypT ($P = 0.493$), ypN ($P = 0.154$), tumor regression grade ($P = 0.901$), or TNM downstaging ($P = 0.217$).

3.3. Clinicopathological correlations with therapeutic tumor response

Table 4 shows the correlations between clinicopathological parameters of patients and tumor response, according to the TNM downstaging and TRG systems, respectively. Downstaging was associated with the cN + clinical stage ($P = 0.001$) and the absence of extracellular mucin ($P = 0.039$). A good treatment response (TRG3) was associated with the absence of extracellular mucin ($P = 0.021$) and the absence of papillary patterns ($P < 0.001$).

3.4. Clinicopathological factors affecting non-downstaging and tumor regression grade

Clinicopathological factors were investigated by multivariate analyses using a logistic regression model (Table 5). The multivariate analyses revealed that older age (≥ 61 years) and HER2 positivity were independent predictors of non-downstaging ($P = 0.028$, odds ratio = 3.837, confidence interval [95% CI] = 1.159–12.703; $P = 0.035$, odds ratio = 5.683, 95% CI = 1.135–28.452; respectively). In addition, poor tumor differentiation and papillary pattern were independent predictors of poor therapeutic tumor response (TRG 1-2 ; $P = 0.026$, odds ratio = 21.921, 95% CI = 1.446–332.37; $P = 0.001$, odds ratio = 38.264, 95% CI = 4.725–309.86; respectively). Therefore, older age (≥ 61 years), HER2 positivity, poor tumor differentiation, and papillary pattern may reliably predict therapeutic failure.

3.5. Clinicopathological correlation with survival

We analyzed the prognostic relevance of HER2 positivity and clinicopathological parameters for OS and DFS. Kaplan-Meier survival analysis showed that patients with TNM downstaging had improved OS (mean ± SD, 103.69 ± 5.31 months) than those with TNM non-downstaging (79.59 ± 8.58 months, $P = 0.045$). However, TNM downstaging was not associated with DFS ($P = 0.177$). A good treatment response was statistically correlated with increased OS ($P = 0.005$) and DFS ($P = 0.017$). No significant associations were observed between HER2 positivity and OS ($P = 0.524$) or DFS ($P = 0.195$, Fig. 1J).

Multivariate analysis confirmed that good therapeutic response was as an independent favorable prognostic factor for OS and DFS ($P = 0.032$, hazard ratio = 0.105, 95% CI = 0.014–0.819; $P = 0.031$, hazard ratio = 0.293, 95% CI = 0.096–0.894; respectively). However, downstaging was not an independent prognostic factor for OS or DFS ($P = 0.111$, hazard ratio = 2.666, 95% CI = 0.799–8.898; $P = 0.238$, hazard ratio = 1.760, 95% CI = 0.689–4.500; respectively).

4. Discussion

The present study investigated the prevalence and role of HER2 positivity as a prognostic or therapeutic marker for locally advanced rectal cancer patients receiving neoadjuvant chemoradiotherapy following surgery. We found that HER2 positivity was a significant predictive factor for non-downstaging, although non-downstaging did not correlate with survival in rectal cancer patients. The possible explanations for the limited prognostic significance of HER2 in rectal cancers are discussed in below.

In the present study, the frequency of HER2 positivity was 17.6% in rectal cancers. This frequency is consistent with the majority of previously reported ranges (14.8–26.7%) of HER2 positivity in rectal cancers, as assessed by IHC and/or *in situ* hybridization [7,24,37]. This frequency of HER2 positivity in rectal cancers seems to be of clinical importance, because of its similarity to the frequency of HER2 positivity in breast cancers (15–25%). HER2-positive breast cancer patients receive therapeutic benefit from the current standard treatment protocol of the humanized anti-HER2 monoclonal antibody, trastuzumab (Herceptin), in combination with chemotherapy. In the present study, multivariate binary logistic regression analysis demonstrated that HER2 positivity and older age (≥ 61 years) were significant predictors of non-downstaging, although the Chi-square test did not reach statistical significance for the association between HER2 positivity and downstaging. However, this poor downstaging was not associated with a poor outcome, which may imply that HER2 positivity in rectal cancer is unlikely to be related to survival. *In vitro* studies have shown that HER2 positivity is associated with therapeutic resistance, but not with survival [6]. Another preclinical study using colorectal cancer cell lines exposed to 5-FU and 2 Gy of radiation also demonstrated that HER2, STAT3, RASSF1, and DOK3 are molecular biomarkers of the response to chemoradiotherapy, but not of survival [32].

The prognostic role of HER2 positivity in rectal cancer patients is still controversial. There is an equal amount of data showing a positive correlation as there is showing no correlation between HER2 positivity

Table 4
Clinicopathological correlations with tumor response according to TNM downstaging and TRG system.

	TNM staging		*P* value	Tumor regression grade		*P* value
	Down-staging n = 42 (%)	Non-downstagingn = 32 (%)		Good response (TRG3) n = 33 (%)	Poor response (TRG1-2) n = 41 (%)	
Age			0.050			0.640
≥61	17 (40.5)	20 (62.5)		15 (45.5)	22 (53.7)	
< 61	25 (59.5)	12 (37.5)		18 (54.5)	19 (46.3)	
Gender			0.202			0.923
Male	32 (76.2)	20 (62.5)		23 (69.7)	29 (70.7)	
Female	10 (23.8)	12 (37.5)		10 (30.3)	12 (29.3)	
Tumor site			0.818			0.658
AV ≥ 6cm	21 (50.0)	15 (46.9)		17 (51.5)	19 (46.3)	
AV < 6cm	21 (50.0)	17 (53.1)		16 (48.5)	22 (53.7)	
CEA level at diagnosis			1.000			0.485
CEA ≥ 5 ng/mL	19 (45.2)	14 (43.8)		13 (39.4)	20 (48.8)	
CEA < 5 ng/mL	23 (54.8)	18 (56.2)		20 (60.6)	21 (51.2)	
cT category			1.000			1.000
cT2 & 3	39 (92.9)	30 (93.8)		31 (93.9)	38 (92.7)	
cT4	3 (7.1)	2 (6.2)		2 (6.1)	3 (7.3)	
cN category			0.001*			0.569
cN0	5 (11.9)	15 (46.9)		10 (30.3)	10 (24.4)	
cN+	37 (88.1)	17 (53.1)		23 (69.7)	31 (75.6)	
Tumor size			0.174			0.112
≥5cm	13 (31.0)	5 (15.6)		5 (15.2)	13 (31.7)	
< 5cm	29 (69.0)	27 (84.4)		28 (84.8)	28 (68.3)	
Differentiation			0.070			0.068
Well/ Moderate	40 (95.2)	26 (81.3)		32 (97.0)	34 (82.9)	
Poor	2 (4.8)	6 (18.7)		1 (3.0)	7 (17.1)	
HER2			0.217			0.901
Positive	5 (11.9)	8 (25.0)		6 (18.2)	7 (17.1)	
Negative	37 (88.1)	24 (75.0)		27 (81.8)	34 (82.9)	
Mucin			0.039*			0.021*
Present	8 (19.0)	14 (43.8)		5 (15.2)	17 (41.5)	
Absent	34 (81.0)	18 (56.2)		28 (84.8)	24 (58.5)	
Papillary pattern			0.597			< 0.001*
Present	12 (28.6)	7 (21.9)		2 (6.1)	17 (41.5)	
Absent	30 (71.4)	25 (78.1)		31 (93.9)	24 (58.5)	

AV, anal verge; CEA, carcinoembryonic antigen; c, clinical tumor or nodal staging on CT/MRI before treatment.
* Statistically significant, $P < 0.05$.

Table 5
Clinicopathological factors affecting non-down staging and tumor regression grade by multivariate analysis.

	Non-down staging		*P* value	Poor response (TRG1-2)		*P* value
	OR	95% CI		OR	95% CI	
Age (y) (< 61 vs. ≥ 61)	3.837	1.159–12.703	0.028*	0.382	0.101–1.448	0.157
Gender (Male vs. female)	0.343	0.090–1.301	0.116	0.546	0.129–2.315	0.412
Tumor site (cm)(AV < 6 vs. ≥ 6)	0.503	0.153–1.649	0.257	2.122	0.581–7.759	0.255
CEA level (ng/mL)(< 5 vs. ≥ 5)	1.306	0.428–3.981	0.639	0.417	0.119–1.457	0.170
Tumor size(cm)(< 5 vs. ≥ 5)	0.403	0.098-1.661	0.208	0.336	0.076-1.480	0.149
Differentiation (W/M vs. Poorly)	4.178	0.629–27.729	0.139	21.921	1.446–332.37	0.026*
HER2 (Negative vs. positive)	5.683	1.135–28.452	0.035*	2.786	0.475–16.342	0.256
Mucin (Absent vs. Present)	3.448	0.991–11.994	0.052	3.411	0.851–13.678	0.083
Papillary (Absent vs. Present)	2.337	0.562–9.713	0.243	38.264	4.725–309.86	0.001*

TRG, tumor regression grading; OR, odds ratio; CI, confidence interval; AV, anal verge; CEA, carcinoembryonic antigen; W, well-differentiated; M, Moderately-differentiated. *Statistically significant, $P < 0.05$.

and survival in rectal cancer patients. However, HER2 positivity was not a prognostic factor for survival in the present study. Earlier studies have reported the prognostic relevance of HER2 positivity for cancer-specific survival [7,24]. Nevertheless, more recent studies, including a meta-analysis, have further demonstrated no prognostic relevance of HER2 positivity to both OS and DFS [12,36,37]. In clinical practice, HER2 positivity does not appear to play a pivotal role in influencing the survival of patients with rectal cancers.

Interestingly, we noted a discrepancy between HER2 IHC score and gene amplification. Despite low HER2 expression (IHC 0 or 1+), three samples (7.7%) with IHC 0 and two samples (11.1%) with IHC 1+ showed *HER2* gene amplification. In contrast, despite high HER2 expression (IHC 3+), 1 case (33.3%) did not show *HER2* amplification. Similarly, Sclafani et al. [30] has also described that 7.9% of HER2 IHC-negative rectal cancers have *HER2* gene amplification. In the present study, all *HER2* gene-amplified cases showed low-level amplification (range, 2.1–2.4) and signal clusters were not observed. Our results suggest that high-level amplification is much less common than low-level amplification in rectal cancers, even though *HER2* gene amplification is implicated as one mechanism for HER2 overexpression [13,27]. Only a single previous study has also reported that most colorectal cancers (99%) exhibit low-level *HER2* gene amplification [1]. HER2 expression may also be the result of polysomy of chromosome 17, transcriptional activation, or translational deregulation [13,27]. We

found that polysomy was common in rectal cancers (36.5%). In this context, a previous study has shown that discordance between IHC results and *HER2* gene amplification may partly result from a low-level increase in gene copy number ratio or polysomy [30]. In a neoadjuvant setting, low-level *HER2*amplification correlates with a lower rate of pathological response to trastuzumab-based therapy in breast cancers [2]. Furthermore, heterogeneous *HER2* gene amplification is frequent (> 50%) in bladder and colorectal cancers [21,29]. The prognostic impact of low-level *HER2* amplification is not as significant as that of high-level amplification [2]. Accordingly, low-level *HER2* amplification may not impact downstream pathways enough to influence cell growth, division, and ultimately, patient survival. This may explain the lack of prognostic impact or specific clinicopathological features in HER2-positive rectal cancers.

In 74 paired pre- and post-treatment specimens, the HER2 IHC scores showed moderate agreement, with an overall concordance rate of 68.9%, indicating that HER2 status is retained, even after chemoradiotherapy. Using post-treatment tumor samples from relapsed refractory rectal cancer patients for molecular studies could be potentially useful in the identification of molecular targets, including HER2.

The influence of an extracellular mucinous component on tumor response to neoadjuvant therapy or prognosis is unclear in rectal cancer patients, since the available data are scarce and conflicting [5,18]. The presence of mucin is independently associated with worse outcomes in rectal cancers, but not in colon cancers [11]. However, another study has reported that there is no statistical difference in survival between patients with the conventional tubular subtype and those with the mucinous subtype of rectal cancer, after receiving preoperative chemoradiotherapy [10]. We observed that mucin was absent in the majority of downstaged tumors (81.0%) and tumors with good treatment response (84.8%), but was present in those with no downstaging (43.8%) and poor treatment response (41.5%). Since extracellular mucin dissects through the tumor wall [10], aiding its local extension, tumors producing copious amounts of extracellular mucin may have poor responsiveness to neoadjuvant chemoradiotherapy and adjuvant chemotherapy in colorectal cancers. In addition, the papillary feature tended not to be present in resected specimens from tumors with good treatment response (93.9%), but it was an independent predictor of poor therapeutic tumor response. Therefore, findings related to the mucin pool and papillary features in resected specimens should be recorded in pathology reports, even though it may not influence patient survival.

There may be some limitations in our study. This was a retrospective study with a small number of enrolled patients. It was conducted in a single center that lacked clinical treatment by anti-HER2 therapy in HER2-positive rectal cancer patients. However, because there are currently no established clinically relevant biomarkers for rectal cancers, the relatively common prevalence of HER2 positivity in these tumors may be clinically important. We have shown that HER2 positivity can predict a high risk of non-downstaging in rectal cancer patients who received neoadjuvant chemoradiotherapy following surgery. This suggests that HER2 positivity is a potential candidate for a clinically relevant biomarker predicting therapeutic failure. The discrepancy between HER2 IHC score and *HER2* gene amplification may result from the low level of amplification, which seems unlikely to affect prognosis.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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